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Repeated Vital Imaging Reveals Schwann Cells Induce and Guide Nerve Sprouting and Regeneration of Neuromuscular Junctions

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Repeated Vital Imaging Reveals Schwann Cells Induce and Guide Nerve Sprouting and Regeneration of Neuromuscular Junctions

by

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Dedication

I dedicate this dissertation to my wife, Lu Jin, for her unconditional love and support.

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At the beginning of this dissertation, I would like to acknowledge the following people: my mentor, Wesley Thompson who did not only provide me the opportunity but also taught me how to be a scientist; Flora Love, Jane Lubischer, Hyuno Kang and my friend Lee Sutton for generous help in these years; Yue Li and Micheale Fideler-Mikesh for their comments on the manuscript as well as Chris Hayworth and Yi Zou for sharing their thoughts with me; Mendell Rimer and Bing Zhang for their suggestions for my future as a scientist. I also would like to thank Guoping Feng, Jeff Lichtman and Josh Sanes for exchanging transgenic mice with our lab.

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Terminal Schwann cells (TSCs) normally sit above the endplate and cover the nerve terminal branches with their processes. However, following denervation, they extend long processes beyond the boundaries of the endplate (Reynolds and Woolf, 1992). Previous results conducted as static observations, showed that TSCs play an important role in the restoration of the synapse at the neuromuscular junction after nerve injuries by extending processes (Son and Thompson, 1995a,b). However, we still do not know the precedence of TSCs in muscle reinnervation following nerve injury and what happens to other synaptic components. These questions are addressed in this dissertation.

In Chapter 2, I report that TSCs extend processes in response to denervation and these processes guide regenerating axons to grow beyond the endplate area. Furthermore, Schwann cells also cause formation of polyneuronal

reinnervation by guiding axons over Schwann cell processes and endoneurial tubes. Finally, withdrawal of TSC processes following denervation induces AChR loss long-term following nerve regeneration.

In Chapter 3, I report that TSCs grow long processes at denervated endplates following partial denervation and in some cases these processes interconnect with innervated endplates. Here the TSCs induce and guide terminal sprouts. Schwann cells in endoneurial tubes are able to guide nodal sprouts, suggesting Schwann cells at both nerve terminals and endoneurial tubes can guide nerve sprouting.

Taken together, these results show Schwann cells are important for synapse formation and maintenance during nerve regeneration.

Table of Contents

List of Tables

List of Figures

CHAPTER 1

Introduction

Neuromuscular junctions (NMJs) are the synapses between motor axons and muscle fibers. Because they are relatively simple and accessible compared to synapses in CNS, they are the most studied of all synapses. Vertebrate NMJs have three components and their anatomy is very simple: a single motor axon wrapped by glia cells known as Schwann cells, is extended from a motor neuron in the spinal cord to innervate a muscle fiber. There are two kinds of Schwann cells: myelinating Schwann cells which myelinate motor axons and form nodes of Ranvier and non-myelinating or terminal Schwann cells whose processes and cell bodies "cap" terminal branches of motor axons at NMJs. These processes are closer to nerve terminals (some 0.15 μm) than the terminal comes to muscle fibers. Studies on Schwann cells spanning about 150 years began with observations of axon-like cells under the light microscope by Schwann in 1839. Recent findings support that glia cells play more roles rather than only generating myelin in PNS.

First, they are believed to participate in buffering of ionic concentration through potassium and sodium channels located on the surface of these cells (Kuffler, 1966; Newman, 1984; Barres, 1991). Furthermore, researchers found neurotransmitter receptors on glia cells in vivo and in vitro. These receptors are believed to clean released transmitters and respond to neurons (Cull-Candy and Wyllie, 1991; MacVicar et. al., 1989). Third, glial cell release trophic factors and

these trophic factors provide a nourishing environment for neurons (Marchionni et. al., 1996). Some of these trophic factors have potency in promoting the survival of motor neurons (Henderson et al., 1994) and others are important in promoting the growth of motor neurons or the extension of processes by Schwann cells themselves (Anton et al., 1994). Meanwhile, neurons also support Schwann cells by releasing some factors that modulate Schwann cells. One prominent example is the axonal supply of neuregulin during early development that is required for the survival of the precursor cells that give rise to Schwann cells (Dong et al., 1995). This supply of neuregulin is also important for the survival of Schwann cells in the neonatal animal. Following denervation in the neonate, the terminal Schwann cells die but can be rescued by exogenous supply of neuregulin (Trachtenberg and Thompson, 1996). Thus, it appears that there are reciprocal, trophic interactions between motor neurons and Schwann cells like those proposed by Verdi et al. (Verdi et al., 1996) for sympathetic neurons and their glial cells.

However less attention was specifically paid to terminal Schwann cells until recently, evidence was obtained showing that terminal Schwann cells appear to participate actively in the maintenance and repair of NMJs (Son et. al., 1996). In normal muscles, terminal Schwann cells sense synaptic transmission taking place at NMJs through receptors binding acetylcholine and adenosine released by motor neurons (Robitaille 1995; Robitaille et al., 1997). Moreover, terminal Schwann cells change their gene expression in response to changes in neuro-transmission (Georgiou et al., 1994). Furthermore, these glia cells are able to

"talk" back to neurons by modulating synaptic transmission in intact muscle tissue preparations (Robitaille, 1998). Compared to the terminal Schwann cells in normal muscles, terminal Schwann cells undergo immediately morphological changes in response to denervation and paralysis, suggesting these glial cells might play new roles in synaptic restoration following nerve injury (Son and Thompson, 1995b).

In response to denervation, terminal Schwann cells extend processes and these processes guide regenerating axons to grow beyond the NMJ area

Terminal Schwann cells do not only regulate synaptic activities in normal muscles, but also play critical roles in restoration of synapses following nerve injury. In 1992, Reynolds and Woolf reported that terminal Schwann cells extended long processes and even a network of processes away from synapses in response to denervation. Even though they did not believe that this growth could play a role in nerve regeneration because the growth appeared to be random, more recent evidence showed that the growth of TSC processes was crucial to nerve regeneration. Not only does denervation cause TSC to grow processes but also blocking neurotransmission with application of botulinum toxin to muscles (Son and Thompson, 1995b). Therefore, terminal Schwann cells are able to sense neuronal activities and respond to denervation and paralysis.

But how does this terminal Schwann cell process function in nerve regeneration? During nerve regeneration, regenerating axons follow old Schwann cell tubes previously associated with axons, leading to individual endplates (Fig. 1.1 panel A-C). Normally, regenerating axons continue to grow instead of

stopping in endplate area and form so-called “escaped fiber”. These fibers are always found to be associated with Schwann cell processes (Son and Thompson, 1995a). So the question is: Do Schwann cell processes precede escaped fibers? Based on static observations, Son and Thompson (1995a) inferred that Schwann cells preceded escaped fibers.

Terminal Schwann cells guide the growth of regenerated axons between synaptic sites

Following nerve regeneration, some endplates are reinnervated by escaped fibers and in some cases by multiple axons: so called “polyneuronal reinnervation”. Interestingly, these escaped fibers (or escaped fiber bridges) were found always associated with Schwann cell processes interconnecting two synaptic sites (Schwann cell bridges) (Son and Thompson, 1995a). Moreover, they found escaped fibers that were on these Schwann cell bridges but did not interconnect two endplates in many cases. Therefore, they suggested that reinnervation by escaped fibers was guided by Schwann cell bridges (Son and Thompson, 1995a) (Fig. 1.1 C-D). Furthermore, Schwann cells rarely form bridges between denervated endplates, but primarily between denervated endplates and the endplates remaining innervation in the muscles following partial denervation. This result implies that some special tropic factors affect growth of Schwann cell processes (Love and Thompson, 1999).

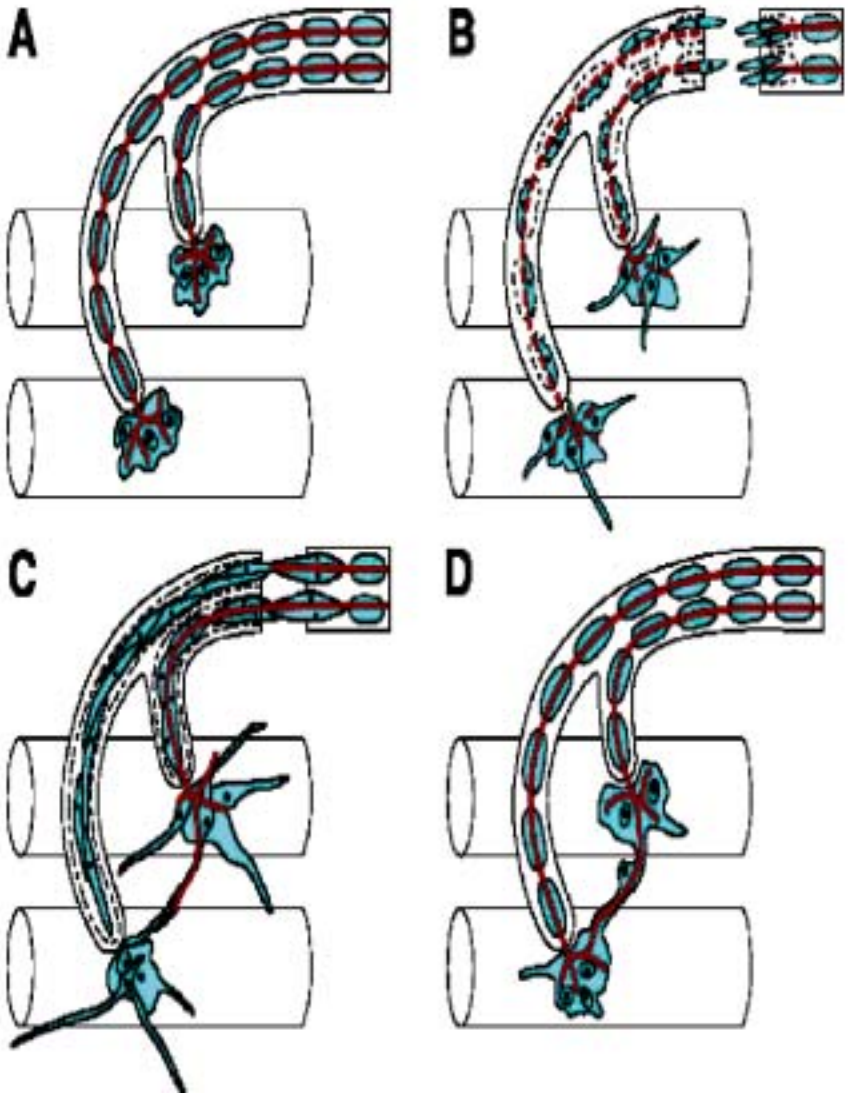

Fig. 1.1: The proposed role of Schwann cells in regeneration of peripheral nerves and muscle reinnervation.

(A) The normal innervation of two muscle fibers by an intramuscular nerve containing two myelinated motor axons (in red) each forming a neuromuscular junction. Terminal Schwann cells (in blue) cover the neuromuscular junctions. In

(B) the nerve has been resected and some of the early consequences are shown. The axons have degenerated distal to the lesion site (indicated by dashed red line). The myelin coating of the former axons is disintegrating and the myelinating Schwann cells (in blue) have begun to extend processes within the basal lamina (endoneurial) tubes of the nerve. Schwann cell processes extend from the nerve on both sides of the lesion. The terminal Schwann cells have begun to extend processes over the muscle fibers .**(C)** The Schwann cells of the nerve have formed a bridge across the lesion site and one axon has regenerated across this site. Processes of the terminal Schwann cells have extended from each endplate and formed fascicles which now interconnect the two endplates. A regenerating axon arriving at the endplate to the right has reinnervated this fiber and is extending beyond the endplate (that is, `escaping') by growing onto the Schwann-cell processes. **(D)** The axon in C has grown along the Schwann-cell processes linking the two endplates to reinnervate the endplate on the left. This endplate is polyneuronally innervated since another axon has arrived by regenerating along the endoneurial tube leading to this endplate. (from Son et al., 1996)

Schwann cells guide the formation of terminal sprouts

Following injury to a portion of the nerve innervating muscles (partial denervation), some of the remaining nerve terminals grow nerve sprouts (terminal sprouts) to innervate adjacent denervated endplates, restoring their innervation; the nature of this additional growth of intact neurons is of great interest to neurobiologists studying the development and plasticity of nervous system (Brown, et. al., 1981). Previous observations showed that both partial denervation and blocking synaptic transmission with botulinum toxin could cause terminal sprouting (Brown et. al. 1980). Moreover, Brown and Holland (1979) reported that direct stimulation of all muscles inhibited sprouting, but not in the case of stimulation only of axons remained following partial denervation. These observations suggested that some factors released from denervated muscle fibers, as a result of their inactivity, cause nerve terminals to sprout (Love, 1999 dissertation).

However, the role of TSC in nerve sprouting had been ignored until the 1990s when several researchers reported that Schwann cells were able to respond to denervation and may participate in nerve regeneration. First, Reynolds and Woolf (1992) reported that following denervation, terminal Schwann cells grew an extensive network of processes away from endplate area, and these processes then retracted when axons grew back to endplates. Second, Astrow et. al. (1994) generated an antibody which could identify denervated Schwann cells. Consequently, using this antibody, Son and Thompson (1995b) found evidence that Schwann cell processes induce and guide terminal sprouting by

immonustaining NMJs for Schwann cells and axons at various stages following partial denervation. Additionally, they also found that these Schwann cell processes are commonly from denervated Schwann cells. They then transplanted denervated nerve which contained denervated SC tubes to normal muscles and found these Schwann cell tubes induced terminal sprouts from intact nerve terminals. These results implied that Schwann cells were able to induce and guide terminal sprouts (Son and Thompson, 1995b). Third, the growth of Schwann cell processes from denervated endplates does not appear random, but to preferentially contact innervated endplates, suggesting that denervated Schwann cell respond to signals present at innervated endplates (Love and Thompson, 1999). Additionally, formation of Schwann cell bridges which may induce and guide terminal sprouts is activity-dependent (Love et. al., 2003).

Taken together, previous results showed that Schwann cells responded to denervation by growing long Schwann cell processes towards innervated endplates and formed Schwann cell bridges; these bridges induced and guided formation of terminal sprouts, suggesting that Schwann cells are able to sense synaptic activity and restore NMJs following partial denervation (Fig. 1.2). However, there are many issues that remain unknown about roles Schwann cells play in normal muscles and in muscles after nerve injury. First, are terminal Schwann cells morphologically dynamic or stable over time at normal muscles? Can we find morphological evidence corresponding to physiological changes in Schwann cells in normal muscles? Second, almost all previous studies concerning guidance by Schwann cells of nerve sprouting were conducted as static

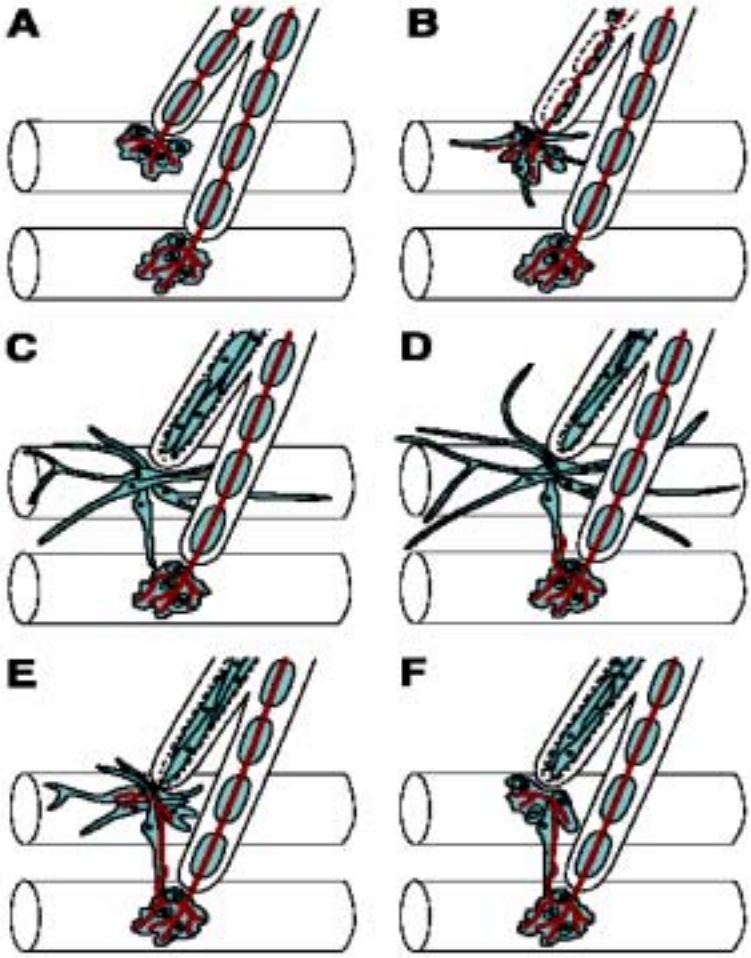

Figure 1.2: The proposed role for Schwann cells in inducing and guiding nerve sprouting in partially denervated muscle.

(A) Two myelinated axons (in red) innervating two muscle fibers. Terminal Schwann cells (in blue) cover the nerve terminal processes at each junction. **(B)** Partial denervation of the muscle injuries to the axon that innervates the top

muscle fiber. This axon begins to degenerate (indicated by dashed red line) and the terminal Schwann cells at its endplate begin to extend processes. In **(C)**, one of the processes extended from the Schwann cells at the denervated junction on the top muscle fiber has reached the nerve terminal remaining on the bottom muscle fiber. In **(D)**, contact with the Schwann cell induces the nerve terminal on the bottom muscle fiber to sprout. The sprout is guided in its growth by the Schwann-cell process. In **(E)**, the nerve sprout has grown to reinnervate the denervated endplate, and the terminal Schwann cells here have begun to retract their processes. **(F)** The consequence of these events: the denervated muscle fiber is now innervated by a sprout arising from the fiber that remained innervated following the partial denervation. (from Son et al. 1996)

observations of muscles dissected from animals at various stages following nerve injury. Therefore, these studies did not prove the precedence of nerves vs. Schwann cells. Direct observations are needed to illuminate the order of Schwann cell's guidance. The best way to answer these questions is to repeatedly image NMJs in living animals. Moreover, rather than just confirming these studies, vital imaging should enable us to learn more about Schwann cell's functions in nerve regeneration and related changes in other components, i.e. Acetylcholine receptors (AChRs) and axons following nerve injury. For example, using vital imaging, we can address Schwann cell's roles in escaped fiber retraction, maturation of reinnervation by nerve sprouts and escaped fibers, reinnervation pattern changes during nerve regeneration, AChR loss and addition following nerve regeneration and so on. Finally, do myelinating Schwann cells have the ability to induce nerve sprouts from nodes of Ranvier (nodal sprouts) as terminal Schwann cells do in terminal sprouting? In vitro approaches might not answer the question since it is almost impossible to identify nodal sprouts from original axon branches. Again, vital imaging may clarify these events.

Vital imaging of neuromuscular junctions in mice using fluorescent labels for nerves and for the AChRs in the muscle fibers had been shown to be feasible by Lichtman, Magrassi and Purves (1987) and had been exploited with great success by Lichtman and his colleagues (Rich and Lichtman, 1989; Walsh and Lichtman, 2003). Our lab generated transgenic mice expressing green fluorescent proteins (GFP) in Schwann cells; and these mice were crossed with mice expressing blue fluorescent proteins (cyan fluorescent protein or CFP) in their

motor neurons (one of the lines of thy-1 promoter-CFP transgenic mice prepared by Feng et al. in 2000). By labeling the junctions with low, non-blocking concentrations of rhodamine-conjugated bungarotoxin, a snake venom component that binds with high affinity to AChRs, and by observing the muscle with appropriate fluorescence filters, we were able to visualize all the components of the junction: the Schwann cells and their processes are green, the axon and their terminals blue, and the postsynaptic AChRs red. By repeatedly imaging the same neuromuscular junctions in a number of animals labeled in this fashion, we were able to observe the terminals and their Schwann cells (both the cell bodies and their processes) as well as the postsynaptic receptors in normal muscles and muscles following nerve injury.

This dissertation documents the roles Schwann cells play in nerve regeneration and nerve sprouting following nerve injury, and consequent changes in NMJs by using vital imaging to repeatedly observe NMJs in the muscles of double transgenic mice.

CHAPTER 2

Schwann cell Processes Guide Regeneration of Axons and Induce Loss of Postsynaptic Components during Peripheral Nerve Regeneration *in vivo*

ABSTRACT

The terminal Schwann cell processes that cover the neuromuscular junction sense neuromuscular transmission and modulate synaptic activities in normal muscles (Son et al., 1996; Robitaille, 1998). Recent researches show the terminal Schwann cell participates in the maintenance and repair of neuromuscular junctions. Reynolds and Woolf (1992) found that in response to muscle denervation, the terminal Schwann cell extends long processes. Based on static observations of rat neuromuscular junctions, Son and Thompson (1995) proposed that Schwann cell processes extended following nerve crush guide regenerating axons to grow beyond endplates, generating so called "escaped" fibers. Further, they found that some Schwann cell processes formed Schwann cell bridges interconnecting two endplates and these Schwann cell bridges guided escaped fibers to innervate adjacent endplates. These results however were from static observations; direct evidence is necessary to better understand Schwann cell functions during nerve regeneration. Repeated vital imaging developed by Lichtman and his colleagues (Lichtman et al., 1987) on double transgenic mice allows us to better study dynamic processes of this guidance and more Schwann cell functions during nerve regeneration through direct observation. In these

double transgenic mice, Schwann cells and their processes express GFP driven by S100 promoter and axons express CFP driven by Thy promoter. Our results agree with previous observations: Schwann cells extend processes in response to denervation and these processes guide regenerating axons to extend from endplates and in some cases even to innervate adjacent endplates, and then may form polyneuronal innervation on these endplates. Moreover, in some cases escaped fibers grow over Schwann cell bridges to innervate adjacent endplates, they do not stop but rather then grow retrogradely over old Schwann cell tubes even to innervate endplates on other side of old Schwann cell tubes. Therefore, we conclude that Schwann cells are crucial for determining the nerve regeneration pattern.

Previous observations showed loss of a portion of postsynaptic components; the AChRs, occurred following nerve regeneration. Further direct evidence from vitally imaging showed this loss is accompanied by loss of polyneuronal innervation: these AChR changes were proposed to be related to synaptic competition. During synaptic competition, the site vacated by the losing axon is not reoccupied by the remaining axon following beginning of AChR loss of this site (Rich and Lichtman, 1989; Culican SM et 1998). However, our results show Schwann cells are also involved in AChR loss following nerve regeneration in addition to the loss of polyneuronal innervation. Following denervation, Schwann cells and their processes withdraw from some sites of AChRs and regenerating axons follow Schwann cell processes both inside and outside of endplates. Therefore, some portions of AChRs are not occupied by regenerating

axons and may be lost consequently. Meanwhile, we also found other cases of AChR loss following nerve crush: such as withdrawal of axons after reinnervation and extension of escaped fibers. Overall, Schwann cells also change the postsynaptic pattern during muscle reinnervation.

METHODS

Animals

Double transgenic mice were used in most of our experiments. These mice most commonly expressed two, soluble, cytoplasmic fluorescent protein- (FP-) reporters: EGFP (enhanced green fluorescent protein) driven by the S100 promoter in Schwann cells (manuscript in preparation), and CFP (cyan fluorescent protein) driven by the thy-1 promoter in motor neurons (Feng et al., 2000). In a few experiments the mice employed were doubly transgenic for thy-1-CFP and GFP driven by nestin promoter and neural enhancer (Li et al., 2003 and manuscript in preparation). These GFP-nestin mice mirror the expression of nestin itself in Schwann cells: nestin is undetectable by immunostaining in Schwann cells in intact nerves and at innervated junctions, but is rapidly up-regulated following denervation. All mice were derived by inbreeding of C57 BL6 and DBA strains and no effort was made to breed to minimize differences in genetic background. Genotyping of FP-mice was initially by Southern blotting or PCR analysis but in later experiments was accomplished by observing the mice (or a small punch from the ear) under a fluorescence dissecting microscope (manuscript in preparation). In most cases mice were bred to homozygousity,

but most animals used in the experiments were progeny that bore a single copy of each transgene insertion.

Surgery

Soleus muscles of young adult, FP-mice (2-3 months of age, both sexes) were anesthetized by an initial intraperitoneal injection (6 µl/gm body weight, 17.3 mg/ml ketamine and 2.6 mg/ml xylazine in 0.9 % NaCl sol.) of ketamin/zylazine mixture. During imaging, a second maintenance dose of 4 µl/gm body weight was administered. The anesthesized animal was placed in a prone position on a magnetic, stainless steel plate and secured with rubber bands attached to its limbs and to magnets that could be positioned on the plate. The right hindlimb was positioned with its lateral surface uppermost, hair was removed with a depilatory, and a midline incision through the skin was made in the shank. To expose the soleus muscle, it was necessary to make a midline incision into the gastronemius muscle, cutting a few muscle fibers where it inserts on the fascia of the anterior compartment. The soleus muscle and its nerve were exposed by retracting the wound opening and retracting the lateral gastrocnemius posteriorly. The exposed muscle was rinsed with sterile lactated Ringers and immersed in the same fluid. Denervation was accomplished by crushing the soleus nerve twice at a distance 1mm from its entry into the muscle with the tips of # 5 Dumont forceps.

In Vivo Imaging

Imaging of soleus was conducted using modifications of the techniques using by Wigston. (Wigston, 1989). The soleus was exposed as described above for the denervation and the plate to which the animal was attached was secured to the stage with an upright epi-fluorescence microscope (Zeiss Axiotech). Exposure and manipulation of the margins of the incision and the muscle and was accomplished with retractors and manipulators attached magnetically to the base plate. A metal rod with its end flattened to the shape of a small spatula was used to lift the soleus muscle by pressing this spatula to the external surface of the medial side of the shank. A volume of lactated Ringers containing 2 μg/ml of rhodamine-conjugated alpha-bungarotoxin sufficient to cover the muscle was applied for 4-5 min. The muscle was then rinsed with lactated Ringers. A small, oval piece of coverglass was cut from a #0 piece of glass to 2-3 mm in its longest axis and its edges firepolished. This oval coverglass was glued to the end of a small rod held in a magnet-anchored micromanipulator, oriented orthogonal to the light axis of the microscope, and lowered into position so it touched the surface of soleus and depressed and flattened the lateral edge of the muscle. After images were collected, the gastrocnemius muscle was moved back into position and the wound was sutured using 6-0 silk suture. The animal was allowed to recover under a heat lamp and then returned to its home cage.

Immunohistochemistry and In vitro imaging

For some experiments, the muscle was removed after the final imaging session and fixed in 4% Paraformaldehyde in PBS (phosphate buffered saline, pH 7.4) for 40 minutes. The muscle was then rinsed in 3 changes of PBS for 10 min each. A thin layer of fibers was then dissected from the surface of the muscle and mounted in fluorescence mounting medium. The same endplates imaged *in vivo* were located *in vitro* based upon the label for AChR.

We also labeled the junctions with monoclonal anti-SV2 antibody to identify synaptic vesicles and supposedly the active part of the junctions. In this case, the muscle was permeabilized with cold methanol (-20^{o}C) for 10 min and Alex Flour 647-Bungarotoxin (Cy5-Btx) was used to label the AChRs. Soleus muscles were fixed in 4% Paraformaldehyde for 10 minutes and rinsed using PBS for 30 minutes. Muscles were then permeabilized using 100% MeOH for 10 minutes (-20^{o}C) and rinsed for 30 minutes. Muscles were then blocked in the blocking solution which was PBS containing 0.3% Triton X-100, 0.2% bovine serum albumin and 0.1% sodium azide for 30 minutes. Muscles were then incubated in the blocking solution containing primary antibodies overnight at room temperature. The following primary antibodies were used: a monoclonal antibody to the synaptic vesicle protein SV-2 (Developmental studies Hybirdoma Bank, diluted 1:500) to visualize the nerve terminal. AchRs were visualized using Cy5-conjugated α-bungarotoxin. After the incubation in primary antibodies, muscles were then rinsed in the blocking solution for 30 minutes. Muscles were then incubated in the blocking solution containing secondary antibodies for 1

hour. The secondary antibody for SV-2 was fluorescein-conjugated sheep $F(ab')_2$ fragment anti-mouse (Sigma, F-2266), diluted 1:100. After the incubation in secondary antibodies, muscles were rinsed in PBS for 30 minutes.

Microscopy and Image Capture

The neuromuscular junctions were imaged using a Zeiss Axiotech with water immersion objectives (10X 0.3NA; 40X 0.75NA; 63X 0.9NA) or, using the Leica MDRX epi-fluorescence microscope with Nikon water immersion objectives (10X 0.3NA; 40X 0.8 NA). Illumination from a 100 watt mercury source was attenuated by neutral density filters (most commonly transmittance 1-10% for TRITC, 1-25% for GFP, and 1-50% for CFP). Various filters and dichroics (Chroma) were inserted for illuminating and viewing the different fluorochromes: TRITC 535/50x, 565LP, 610/75m; GFP 495/30x, 515LP, 535/30m; CFP 436/20x, 455DCLP, 470/40m. The filters and dichroics for GFP and CFP were designed to reduce the cross-over in fluorescence excitation and emission for these two FPs at some cost of signal intensity; they worked very well except for cases where the GFP fluorescence was much brighter than that for CFP, which happened rarely in our experiments.

The images were acquired using one of two sensitive CCD cameras, a Princeton MicroMax 5MHz (Roper Sci.) or a Photometrics Coolsnap HQ (Roper Sci.). Macintosh computer with IP Lab software (Scanalytics, Fairfax, VA) was used to control the camera and an electronic shutter in the illumination path.

Images were commonly collected with 1 sec exposures with the cameras set to bin at 2X2 (image of 650 X 515 pixels).

RESULTS

Repeated images of the same endplates show that the AChR, Schwann cell and nerve are morphologically constant over time

Generation of transgenic mice expressing both GFP in their Schwann cells and CFP in their motor axons allows imaging of Schwann cells, motor nerve terminals, and acetylcholine receptors (via application of rhodamine-conjugated bungarotoxin) at neuromuscular junctions in living animals. However, the usefulness of these animals would be severely limited if either transgene expression or imaging itself induced changes in muscle innervation. To examine these issues, a number of control experiments were first performed.

First, images were collected from 24 neuromuscular junctions in 5 soleus muscles in doubly transgenic, adult mice. When imaged a second time 30 days later, none of the junctions showed changes in the pattern of nerve branching, the direction from which the nerve entered the synaptic site, or a production of nerve sprouts. An example is shown in Fig. 2.1. Furthermore, with a few exceptions noted below, there was no change in the number or position of Schwann cell somata visible over the junction. All but 2 endplates in soleus muscles showed no change in the AChR-rich synaptic gutters that lay underneath the nerve terminal branches. These observations suggest, in agreement with observations of Lichtman and colleagues, that neuromuscular junctions in adult mice generally do

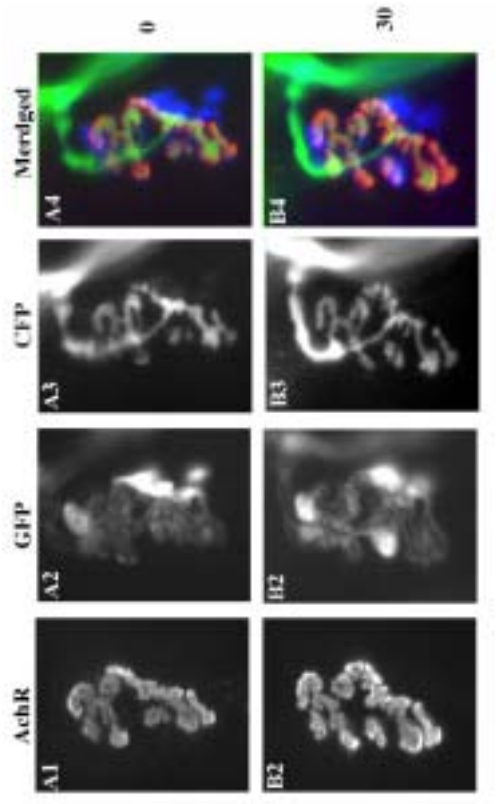

Figure 2.1: Vital repeated imaging shows that AChRs, SCs and nerves are persistent at NMJs of normal muscles

Acetylcholine receptors (AChR), Schwann cells (SCs), and nerves were viewed prior to denervation and then over the course of 30 days. In the merged images (A4 and B4), AChRs, SCs and nerves have been colored red, blue and green, respectively. New rhodamine-bungarotoxin was added on days 0 and 30. The first images were collected on day 0. A single axon wrapped by terminal SCs and their processes, enters the endplate and divides into 3 branches that cover all the acetylcholine receptors (penal A). When the next image was collected on day 30 days later, changes in the pattern of nerve branching, the direction from which the nerve entered the synaptic site, or a production of nerve sprouts (penal B). Bar 20 μm

not change their gross, light microscopic morphology over such time periods, and here we extend these observations to the synaptic glial coverings. We did however observe changes in a small number of the junctions. 2 of the junctions showed a minor loss of AChR areas amounting to less than 5% of the total area. The loss appeared to be the appearance of a discontinuity in a previously continuous synaptic gutter.

We next extended these observations to include more viewing times. Fifteen junctions in 4 adult soleus muscles were imaged a minimum of 4-5 times over the course of 30 days. Thirteen of these junctions showed no alteration in the morphology of the synaptic connection. An example is shown in Fig. 2.2. One of these showed a minor loss of AChR near the center of the endplate. One of these junctions showed the appearance of a long Schwann cell process that ran along the muscle fiber parallel to the long axis of the muscle fiber, but there were no changes in the nerve terminal, growth of axon processes along this Schwann cell, or AChR at this synapse. Such long Schwann cell processes are occasionally seen in animals upon an initial observation (personal observations) and therefore, we do not believe that any of the changes we observed were induced as a result of our observations. We conclude therefore, that endplates can be observed repetitively in transgenic animals expressing GFP and its variants in motor neurons and Schwann cells, as in the experiments reported below, without induction of changes in the synapses as a consequence of either imaging or transgenesis.

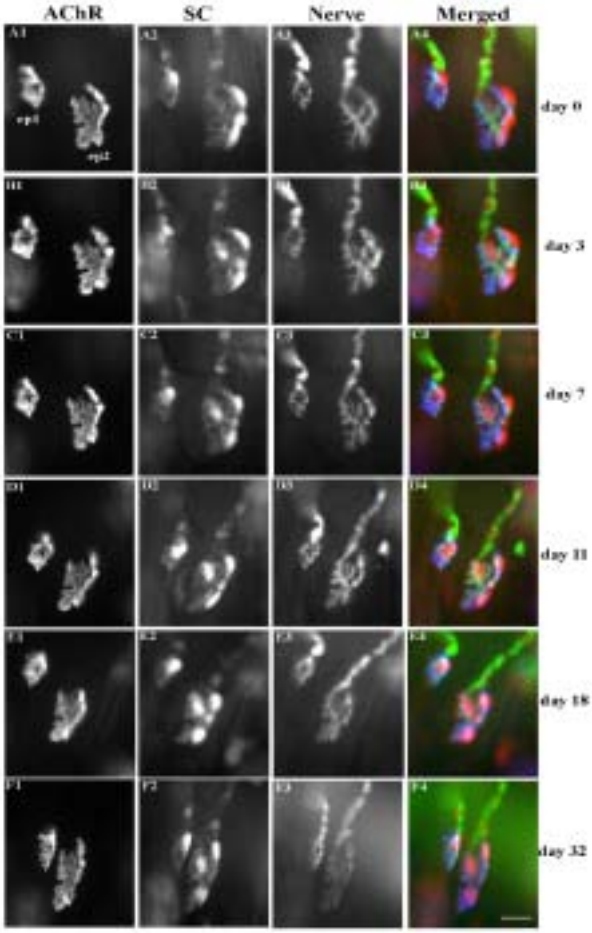

Figure 2.2: Acetylcholine receptors, Schwann cells and nerves are stable at neuromuscular junctions during repeated imaging of normal muscles in vivo.

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at two endplates (ep1 and ep2) were viewed repeatedly over the course of 32 days in a normal muscle. In the merged images (A4, B4, D4, E4 and F4), AChRs, SCs and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day 0, 11 and 32. In the last images, these two endplates were examined in vitro on day 32 after sacrifice of the animal and removal and fixation of the muscle. No AChR loss or addition, SC process extension or nerve sprouting is detected from day 0 to day 32. Bar 20 µm

Schwann cells at normal junctions extend and retract short processes

During the course of these control experiments, we did encounter a number of junctions where Schwann cells extended short processes from the synaptic site beyond the AChR. These Schwann cell processes were quite short (on average 1.1 ±0.5 μm) and were found in both soleus and sternomastoid muscles (Figure 2.3 Panel A). These short processes were obviously different from the numerous, long Schwann cell extensions found following denervation (see below). In no case were these short processes associated with extensions from the nerve terminal. The incidence of these short Schwann cell processes was ca. 47% of the endplates in sternomastoid muscles (42 of 90 endplates in 32 muscles) and ca. 50% of the endplates in soleus muscles (56 of 98 endplates in 16 muscles). To determine whether these short extensions might be induced by the surgery or the imaging itself, we examined muscles taken acutely from control, GFP-transgenic or wild type animals. These muscles were labeled with antibody to S100 to visualize Schwann cells. Short Schwann cell processes were found on 49% of endplates (n=43) in 2 transgenic animals and 43% of endplates (n=30) in 2 wild type animals (Figure 2.4). We conclude that these short processes are normal features of terminal Schwann cells at mouse NMJs. Other observations suggest that these short Schwann cell processes are dynamic (Figure 2.3). Some short Schwann cell processes, present at a first imaging were absent when a second image was collected, and some junctions presented such processes that were absent at the time of the first viewing. These observations suggest that

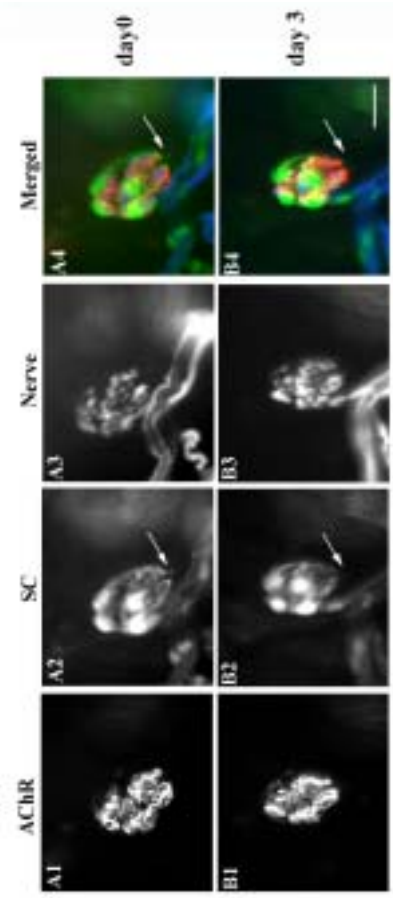

Figure 2.3: In vivo images show Short Schwann cells processes disappeared upon a subsequent view in a normal muscle.

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at a single endplate were viewed repeatedly over the course of 3 days in mornal soleus muscle. In the color images (A4 and B4), AChRs, SCs and nerves appear as red, green and blue, respectively. AChR, nerve terminal and Schwann cell bodies and processes covering the nerve terminal are morphologically unchanged over time, but short processes (arrow) extended by Schwann cell beyond AchR and nerve terminal have disappeared. Bar 20 μm

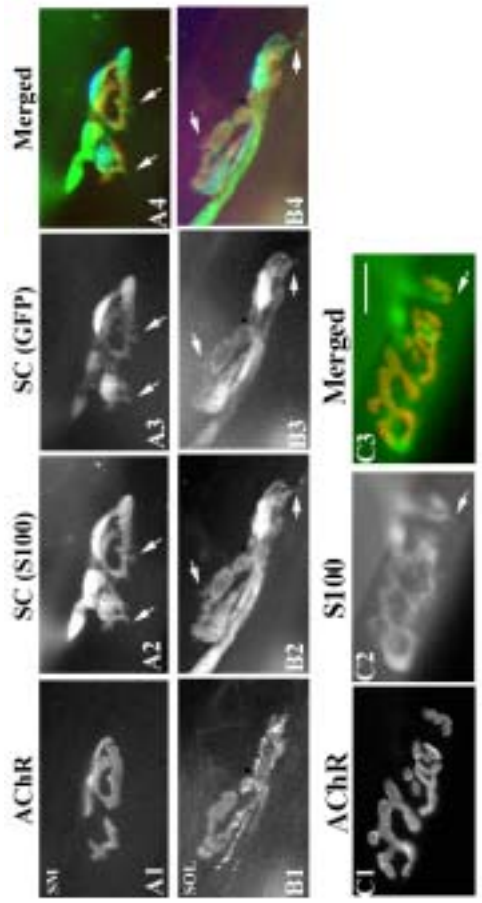

Figure 2.4: Short Schwann cells processes also can be viewed by immunocytochemistry.

AChRs, Schwann cells (SCs) were imaged in a soleus and a sternomastoid muscle in vitro. Schwann cells (SCs) are labeled by S100 antibody (S100) in mice expressing GFP (GFP) driven by S100 promoter (Panel A and B) and wild type mouse (Panel C). Panel A shows that Schwann cells extend short processes (arrow in A2, A3 and A4) in a sternomastoid muscle (SM). The same result from a soleus muscle is in panel B (arrow in B2, B3 and B4) (SOL) and a soleus muscle of a wild type mouse (arrow in C2 and C3). In color images A4 and B4, AChRs, S100 labeling and GFP appear as red, green and blue, and AChRs and S100 labeling were colored red and green in C3. S100 protein antibody staining is consistent with GFP (panel A4 and B4). Bar 20 μm

Schwann cells might be constantly probing the environment around the synaptic site and withdrawing these processes if they are not somehow stabilized. (Note: Some of in vivo work on sternomastoid muscles was done in collaboration with Hyuno Kang.)

In response to denervation, Schwann cells extend processes into perijunctional space

Several investigators have now reported that terminal Schwann cells grow processes from endplates following denervation (Reynolds and Woolf, 1992; Astrow et al., 1994). To investigate the extension of Schwann cell processes in our transgenic mice, we resected the soleus nerve in 3 mice and examined the muscles in vitro 4 days later. Of 88 endplates examined, 73 had processes extending from their terminal Schwann cells. The orientation of this growth was mostly along the long axis of the muscle fibers and, in agreement with the observations of Love and Thompson (1999), these Schwann cell processes in these denervated muscles tended not to grow into contact with adjacent endplates. An example is shown in Fig. 2.5 (Panel A and B).

To examine how quickly following denervation Schwann cells began to extend these processes, we crushed the soleus nerve and examined 6 endplates in two soleus muscles before and then 1, 2, 7, and 10 days following nerve crush. CFP labeling in the motor axons was mostly lost between day 1 and day 2 in these 6 endplates. Extension of processes by Schwann cells was observed in one endplate 1 day after denervation, two additional endplates had extended processes by 2 days after denervation, and all of the endplates had grown processes 7 days after denervation. We also collected repeated images of some 32 Schwann cell

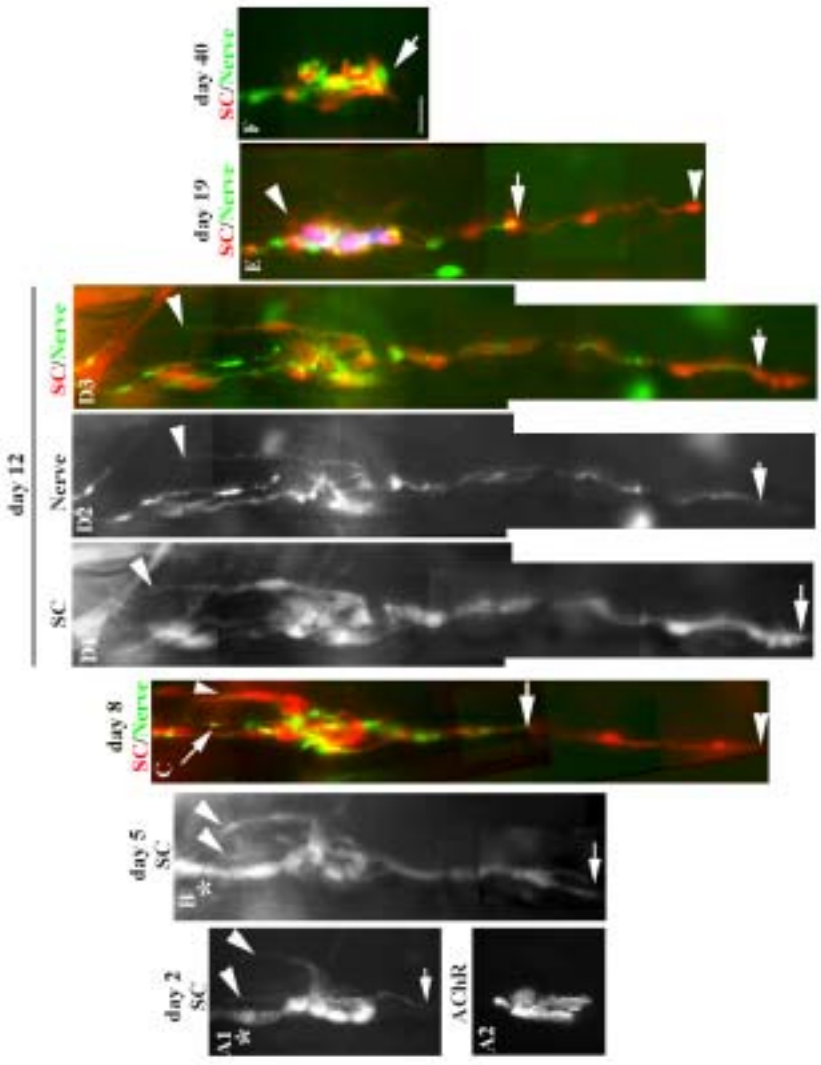

Figure 2.5: Escaped fibers follow SC processes during nerve regeneration and are subsequently withdrawn following reinnervation

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at a single endplate were viewed repeatedly over the course of 40 days following nerve crush. In the color images (C, D3, E and F), SCs and nerves appear as red and blue, respectively. New bungarotoxin was applied on day 2, 8, 19 and 40. When the first images were collected on day 2 following nerve crush, there was no nerve

present (image not shown), but the SC has extended at least 3 SC processes away from the endplate (2 of which are labeled with arrowheads in B; another in B is labeled with an arrow that indicates the furtherest extent of this process from the endplate). On day 5 the nerve has still not returned to this endplate (image not shown); however, the SC processes (arrowheads and arrow in B) have become longer and thicker (compare distance from endplate of arrow in B with that in A1). When the endplate was imaged on day 8, an axon had regenerated and reinnervated it (green label in C), entering through the old Schwann cell tube (asterisks in A1 and B). The reinnervating axon extended escaped fibers out onto two of the SC processes (those marked with the small arrow and the bottom arrowhead in C). On day 12, two observations are noteworthy. First both the bottom SC process and the corresponding escaped fiber have become longer, and the SC process is still longer than the escaped fiber (arrow in D1, D2 and D3). Second, the third escaped fiber (arrowhead in D2) follows a preexisting SC process (right arrow in B and top arrowhead C). Again, the SC process is longer than the escaped fiber (arrowhead in D3). On day 19, SC processes and escaped fibers have withdrawn, the top two completely while the bottom one has become shorter. Note that bottom escaped fiber has withdrawn more extensively than the SC process (compare arrow and arrowhead in E). By day 40, the last escaped fiber and the SC process disappear (arrow in F). Bar 20 μm

processes extended from 18 endplates following nerve crush but prior to reinnervation in 7 soleus muscles. Twenty of the processes seen on the first view had grown longer by the second image (growth averaging 8.5 ± 1.4 μm/day), 6 stayed at roughly the same length, and 6 had retracted or become shorter. It is clear from these observations that Schwann cell processes are dynamic. Since our observations were concentrated on the longer Schwann cell processes which were stable, the observations could easily underestimate the extent of this dynamism.

Regenerating axons extend beyond endplates by following Schwann cell processes that grow during the period of reinnervation

Following denervation by crushing the nerve, axons regenerate to the sternomastoid or soleus muscles reasonably quickly; the first axons return in less than a week. Previous observations suggested that regenerating axons grow along the Schwann cell processes extended during denervation (Son and Thompson, 1995, O'Malley et al., 1999), forming "escaped fibers".

Our vital imaging showed that axons arrived at most endplates by growing down the old endoneurial tube ("old Schwann cell tube") that wrapped the axon previously innervating each site (see exceptions described below) (Tello, 1907, Ramon y Cajal, 1959, Rich and Lichtman, 1989, Nguyen et al., 2002). Axons began to reoccupy the synaptic site, but before this reoccupation was completed, they often extended processes beyond the old synaptic site, i.e. formed "escaped fibers" (Gutmann and Young, 1944). Escaped fibers were always found in association with Schwann cell processes. Observations of 20 soleus muscles during the period of reinnervation showed that 75 of 84 endplates formed escaped

fibers, all of which were associated with Schwann cell processes. To assess the possibility that these escaped fibers were growing in association with pre-existing Schwann cell processes, we carried out detailed examinations on 38 escaped fibers at 22 endplates imaged repetitively in 9 soleus muscles. Of these 38 escaped fibers, all but 4 were found to be growing along a Schwann cell process extended from the endplate prior to reinnervation. An example is shown in Fig. 2.5. Since all escaped fibers were associated with Schwann cell processes, the 4 exceptions appeared to have followed Schwann cell processes that grew just prior to reinnervation (but after our last observation) or shortly after reinnervation began. In no case were the escaped fibers observed to be in advance of the Schwann cell processes. These data, together with previous observations, argue strongly that Schwann cells serve as the substrate for formation of escaped fibers during reinnervation of neuromuscular junctions. It is important to note however, it is probably the case that not every Schwann cell process promotes the formation of an escaped fiber. In our experiments we found many cases where there were more Schwann cell processes extending from a synaptic site than there were escaped fibers. While it is formally possible that all Schwann cell processes are transiently occupied by escaped fibers and that some of these escaped fibers are rapidly withdrawn, we do not favor this possibility because observations at shorter, 1 day intervals suggest that some of Schwann cell processes remain unoccupied by escaped fibers.

In some cases both Schwann cell processes and escaped fibers continued to grow during reinnervation of the synaptic site (Fig. 2.5 Panel C and D).

However, even in these cases the Schwann cell processes were consistently longer than the escaped fibers. Sometime after the synaptic site is reoccupied, Schwann cell processes and escaped fibers (unless these escaped fibers have connected with an adjacent endplate (see below) withdraw. Escaped fibers always withdrew faster than the Schwann cell processes (Fig. 2.5 Panel E and F). Therefore, in some cases Schwann cell processes remain in cases where escaped fibers have already withdrawn. We almost never observed a case where the more slowly retracting Schwann cell processes became occupied by another nerve growth, suggesting that even though Schwann cell retraction is slower, some change has occurred that makes this Schwann cell process unattractive for further nerve growth. Alternatively, the branch of the motor neuron innervating this endplate may have switched from a growth mode.

Schwann cells cover AChRs at former synaptic sites immediately following denervation but gradually lose this coverage

Immediately following denervation, terminal Schwann cells, in addition to extending processes, remain in position covering the underlying AChRs. With longer times of denervation, this association deteriorates such that receptor areas are no longer occupied by Schwann cells or their processes (Fig. 2.6). To quantify the extent of this change, we denervated soleus muscles by nerve resection to prevent reinnervation and then examined the extent of receptor coverage by Schwann cells at old synaptic sites at various times later. In these cases, to increase the numbers of junctions examined, the muscles were removed from the animals and examined in vitro. In soleus muscles (n=3), some portion of the

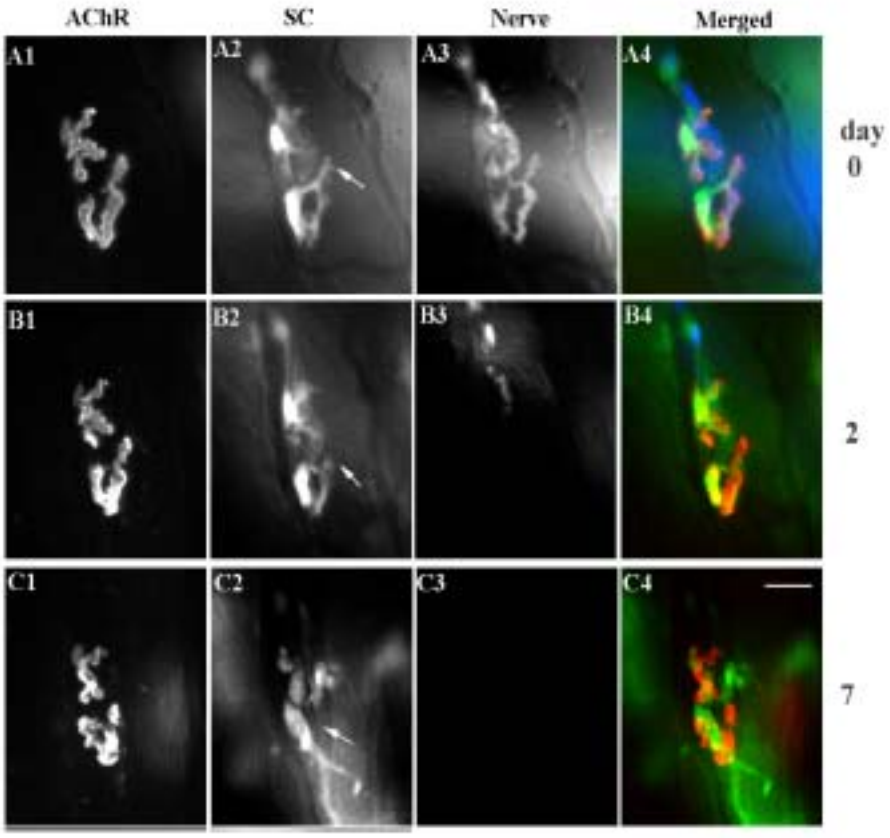

Figure 2.6: Schwann cells cover AChRs at former synaptic sites immediately following denervation but gradually lose this coverage

AChRs, Schwann cells (SCs) and nerves at a single endplate were imaged repeatedly over the course of 7 days following nerve crush. In color images (A4, B4 and C4), AChRs, SCs and nerves appear as red, blue and green, respectively. New bungarotoxin was applied on day 0. At the time before nerve crush, SCs and their processes covered all AChRs (A4). However 2 days following nerve crush, at least one of SC processes began to withdraw and left small portion of the AChR site uncovered (compare arrows in A2 and B2). Furthermore, SC coverage on all AChRs became much less and abundant that whole AChR site on day 7 (compare arrows in B2 and C2). Note: on day 7, the axon did not grow back. Bar 20 μm

AChR site was devoid of Schwann cells at about 62% of the 80 junctions examined 4 days later. On average, 16±1.1% of the synaptic site was not covered by terminal Schwann cells (n=26).

Endplates lose AChR sites as a consequence of reinnervation.

During the course of our imaging we observed a considerable number of cases where endplates lost AChR sites within a month or so of reinnervation. To get some idea of the extent of this loss, we first conducted a series of experiments in which we imaged axons (via CFP fluorescence), Schwann cells (via GFP fluorescence), and AChR (via application of nonblocking concentrations of rhodamine-alpha-bungarotoxin) at junctions in the soleus. We then denervated these muscles by nerve crush and re-imaged the same junctions 30 days later. 13 of 25 endplates in 5 soleus muscles lost receptor sites that exceeded 5% of the total initial area of AChR. These 13 endplates lost on average 17.3±2.7% of their receptor area. An example is shown in Fig 2.7 in panel A-D. In addition 2 of these 25 endplates gained receptor areas and this topic is dealt with below.

We next attempted to verify that these losses were not a consequence of the viewing these junctions with 3 different wavelengths of light or to the transgenes expressed in these double-transgenic mice. As an additional control, we took wild type mice and made an initial set of images from the soleus muscle that was labeled only with rhodamine-bungarotoxin. Under rhodamine fluorescence, we imaged the receptors at a number of endplates that could be viewed en face. We then denervated the muscle by crushing the muscle nerve. Thirty days later these same endplates were viewed again after restaining with

rhodamine-bungarotoxin. We noted any change in the receptor pattern and measured the areas of the receptor sites that were added or lost; the area added or lost was then computed as a fraction of the total area of the AChR site in the original image. We found that during reinnervation 13 of 27 endplates in 4 soleus muscles lost receptor sites that exceeded 5% of the total initial area of AChR. These 13 endplates lost on average 12.6±1.3% of their receptor area. An example is show Fig 2.7 in panel E-F. In addition 2 of these 27 endplates gained receptor areas (the size of the gain 10% and 13%). (see Table 2.1)

From these two sets of experiments, we conclude that receptor loss is a common feature of reinnervation following single nerve crush. Less common, but still present following a single nerve lesion, is the addition of new receptor sites.

Failure of regenerating nerves to reoccupy portions of synaptic sites explains a portion of the loss of receptor sites

One possible explanation for the postsynaptic receptor loss in reinnervated junctions, we reported above, is that axons fail to reoccupy old receptor sites. Balice-Gordon and Lichtman have shown using focal application of blocking doses of bungarotoxin that inactive receptor sites in an otherwise active junction disappear thus the unoccupied and inactive AChR sites in reinnervated endplates would be expected to be eliminated. We did observe a number of cases where failure of reoccupation appeared to account for loss of receptor sites. In 7 soleus muscles, we observed 20 endplates in which we obtained an image before reinnervation, an image within 1-2 days of the beginning of reinnervation, and

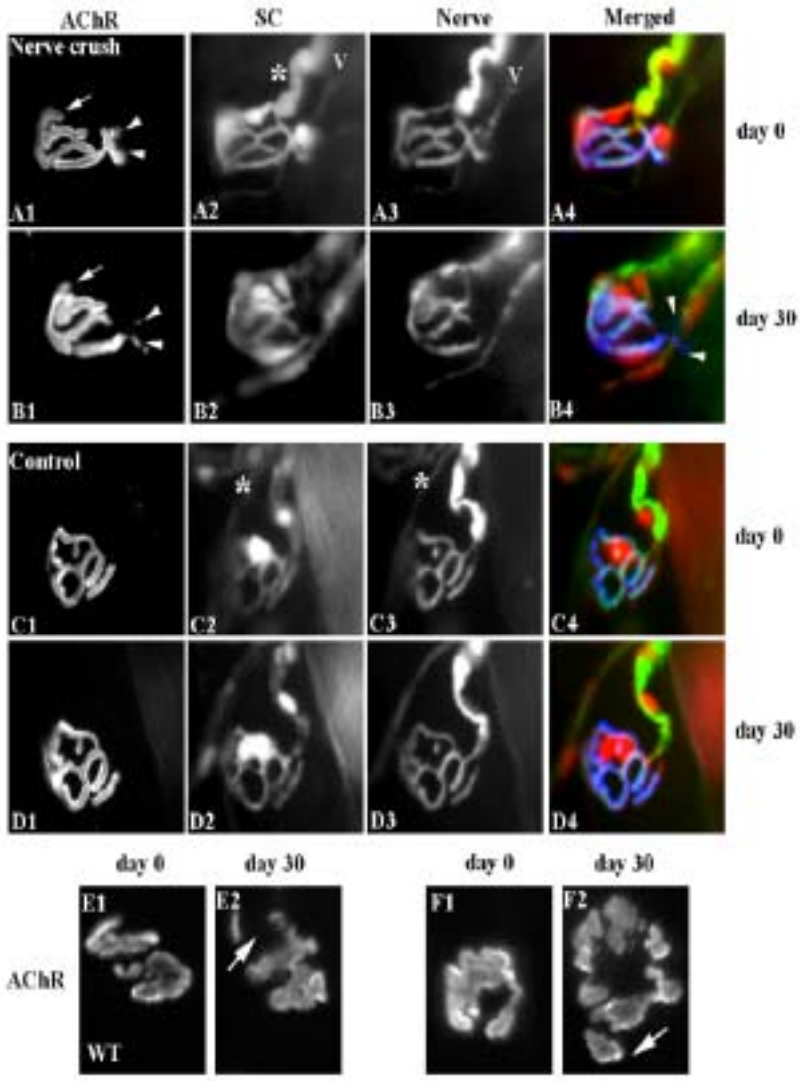

Figure 2.7: Acetylcholine receptor loss and addition take place at reinnervated endplates in the muscles long-term after nerve crush, but not in normal muscle

Panel A-D: Acetylcholine receptors (AChR), Schwann cells (SCs), and nerves were viewed at an endplate prior to denervation and then over the course of 40 days of reinnervation and at an endplate of the normal muscle over the course of 30 days. In the merged images (A4, B4, C4, D4), AChR and nerves have been

colored red and green, respectively. New rhodamine-bungarotoxin was added on days 0, 30, and 40. Nerve crush (panel A and B): When the first images were collected, a single axon enters the endplate and branches to cover all AChRs (panel A). Note there is an axon associated with a nearby SC tube innervating endplates deeper in the muscle ("V" in A2 and A3). When the next images were collected 30 days after nerve crush, an axon reinnervated the endplate by following the old SC tube (asterisk in A2) but did not cover all AChRs (arrowhead in B4). There is AChR loss at the endplate (compare arrow in A1 and B1). Moreover, faintly labeled AChRs which are not covered by either the SC process or the nerve terminal were found at right side of the endplate (compare arrowheads in A1 and B1). These faintly labeled AChRs were lost on day 40 (data not shown). Control (panel C and D): AChR loss such as that described above is not found at the endplate of a normal muscle (compare C1 and D1). Note there is a nearby axon wrapped by SCs that innervates endplates deeper in the muscle (asterisk in C2 and C3).

Panel E-F: AChRs were repeatedly imaged in two soleus muscle in vitro over the course of 30 days in wild type mice following nerve crush. Panel E show AChR loss (arrow in E2) and panel F show AChR addition (arrow in F2) 30 days following nerve crush. Bar 20 μm

Table 2.1 Summary of data of AChR loss 30 days following nerve crush

Animal type	Experiment	Number of animal	Number of endplates	Percentage of endplates with AChR loss	Percentage of AChR loss in individual endplates	Percentage of endplates with AChR addition
Double transgenic mice	Control	5	24	8.3% (2/24)	Less than 5%	
	Nerve crush	5	25	52% (13/25)	17.3±7.5%	8% (2/25)
Wild type mice	Nerve crush	4	27	48% (13/27)	12.6±1.3%	7.4% (2/27)

then subsequent images after reinnervation. For 7 of these 20 endplates we observed, at the initial image, an area of the AChR site that was not occupied by Schwann cells or their processes. Moreover, no nerve or Schwann cell process was ever seen above these sites either early or late in reinnervation. In these cases the bungarotoxin labeling of the unoccupied site dimmed and ultimately disappeared (Fig. 2.8). We also sought additional examples of such loss of unoccupied areas by focusing attention on those endplates observed early in their reinnervation (within a day of the arrival of the regenerating axon) that had an AChR area unoccupied by axons or Schwann cells. We found 29 such endplates in 5 soleus muscles and subsequent images failed to show that these areas ever became reoccupied. An example is shown in Fig. 2.9. The AChR at these sites dimmed in the bungarotoxin labeling and ultimately disappeared.

However, in some cases, some receptor sites initially unoccupied by the nerve and Schwann cells are subsequently reoccupied (Fig. 2.10). Therefore, despite the fact that failure of reoccupation of sites by regenerating axons is an explanation for receptor loss, it is clear that Schwann cells abandon more synaptic sites than lose of receptor. Moreover, in some cases, regenerating axons did not initially occupy AChR sites covered by Schwann cells, but re-occupy these AChR sites following withdrawal of escaped fibers (Fig. 2.11).

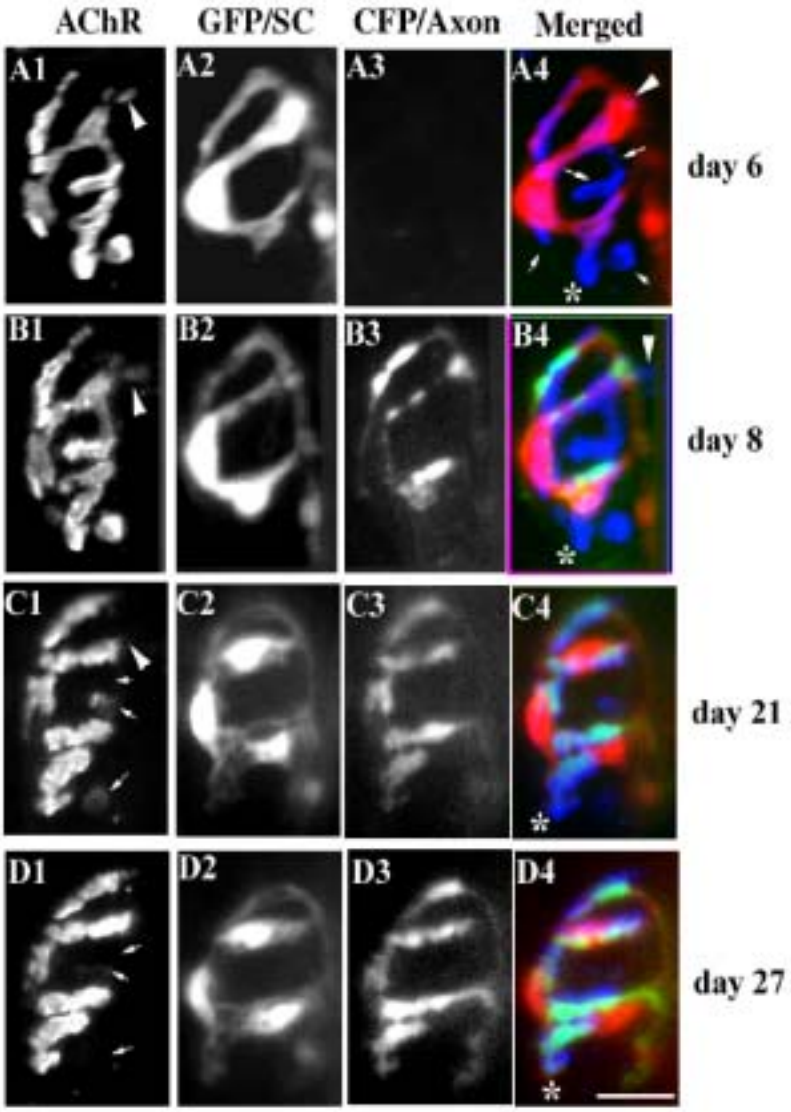

Figure 2.8: **Within endplates, withdrawal of Schwann cell processes from AChR sites during the period of denervation is associated with failure of reinnervation of these sites and the subsequent loss of the receptors from these sites.**

Acetylcholine receptors (AChR), Schwann cells (SCs) and nerves at an endplate were viewed repeatedly during reinnervation over the course of 27 days following

nerve crush. In the merged images (A4, B4, C4, and D4), AChRs, SCs, and nerves appear as blue, red and green, respectively. New bungarotoxin was applied on days 6, 21, and 27. When the first images were collected on day 6, the endplate had not yet been reinnervated as shown by the absence of label in A3. SCs and their processes only partially occupy AChRs (receptor areas uncovered by SCs are indicated by arrows and asterisk in A4). On day 8 an axon has regenerated to the endplate by growing up the old SC tube (most obvious in the lower right side of A2 and B2). At this time, the regenerating axon seems to be confined to areas occupied by SCs on day 6. This means several AChR sites (solely blue areas in B4) have failed to become reinnervated. By day 21 these AChR sites have become faintly labeled or lost (arrows in C1). This loss continues in the images collected on day 27. Note however, that one AChR synaptic site not occupied by Schwann cells and not initially reinnervated (asterisk in B4), does become covered by SC processes and becomes reinnervated escapes elimination. Moreover, one AChR site was covered by a SC body on day 6 (arrowhead in A1 and A4). However, the Schwann cell body in A2 has moved by day 8 and a portion of the receptor area (arrowhead in B4) was left uncovered and is not reinnervated by the regenerating axon at day 8. This receptor area became faintly labeled and disappeared by day 21 (arrowhead B1 and C1). Note the addition of a new SC between day 8 and day 21. Bar 20 μm

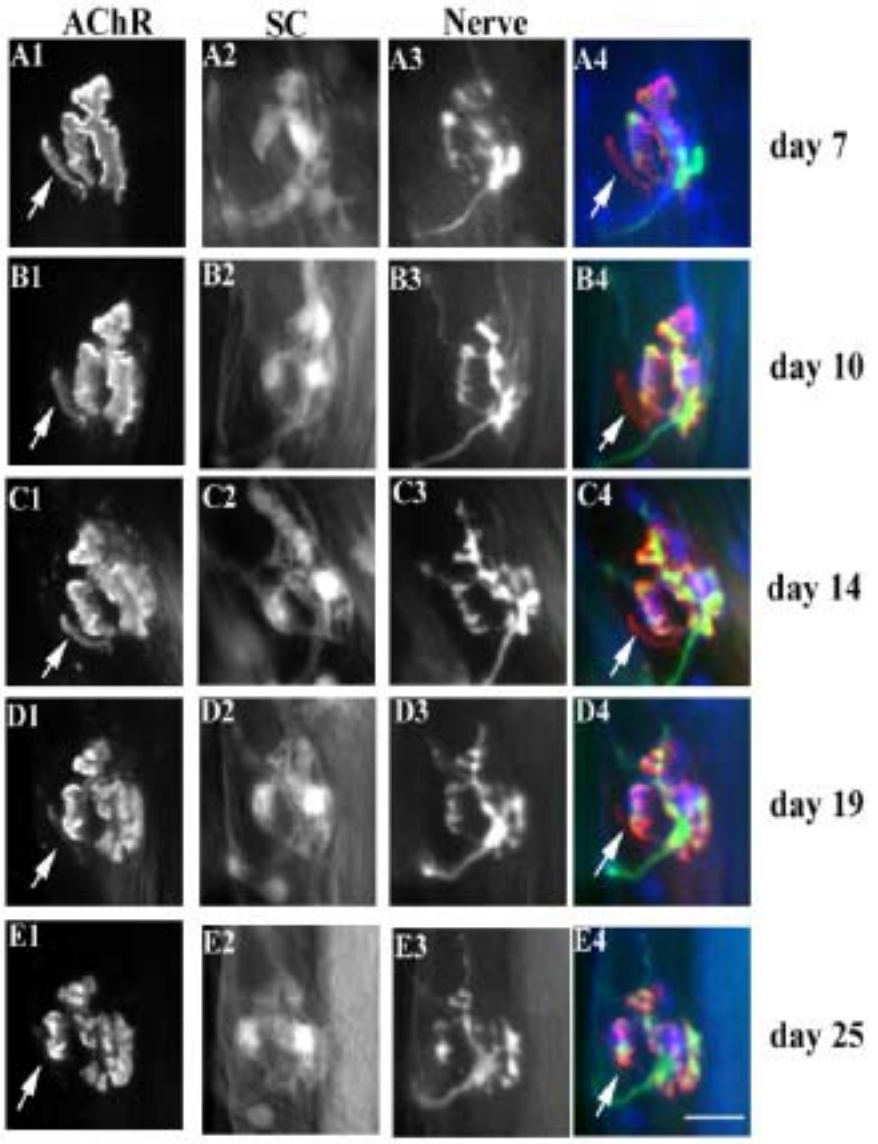

Figure 2.9: Within endplates, AChR loss is induced by inoccupation of Schwann cells and nerves long term following nerve regeneration

Acetylcholine receptors (AChR), Schwann cells (SCs) and nerves at an endplate were viewed repeatedly during reinnervation over the course of 25 days following

nerve crush. In the merged images (A4, B4, C4, D4 and E4), AChRs, SCs, and nerves appear as red, blue and green, respectively. New bungarotoxin was applied on days 7, 14, 19 and 25. When the first images were collected on day 7, the endplate had been reinnervated by an axon from bottom as shown in A3. SCs and their processes and nerve terminals did not occupy one AChR site (receptor areas uncovered by SCs and nerve terminals are indicated by arrows in A1 and A4). On day 10 and 14, this AChR site was still uncovered by SCs and nerves, and it appears that this site are smaller (compare arrows in panel B and C). This receptor area became faintly labeled on day 19 (arrows in D1 and D4) and disappeared by day 25 (arrows E1 and E4). Bar 20 μm

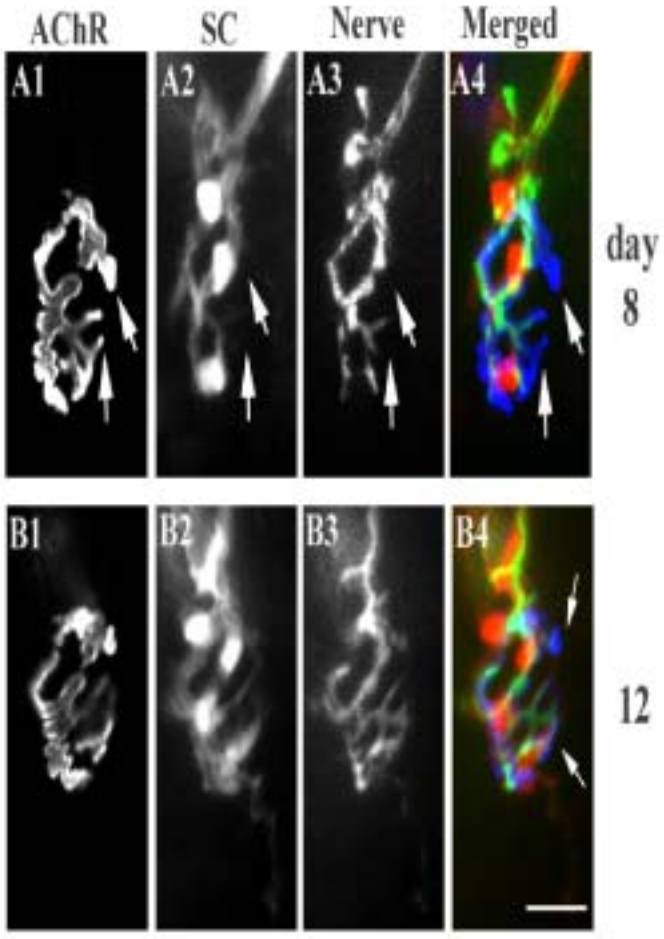

Figure 2.10: Some receptor sites initially unoccupied by the nerve and Schwann cells are subsequently reoccupied

Acetylcholine receptors (AChRs), Schwann cells (SC) and nerves at one endplate were viewed repeatedly over the course of 12 days following nerve crush in a soleus muscle. In the merged images (A4 and B4), AChRs, SC and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day 8 and 12. On day 8, some AChR sites were not covered by SC processes and nerve terminals (compare arrows in panel A). However, these sites were reoccupied by SC processes and terminals 4 days later (arrows in B4). Bar 20 μm

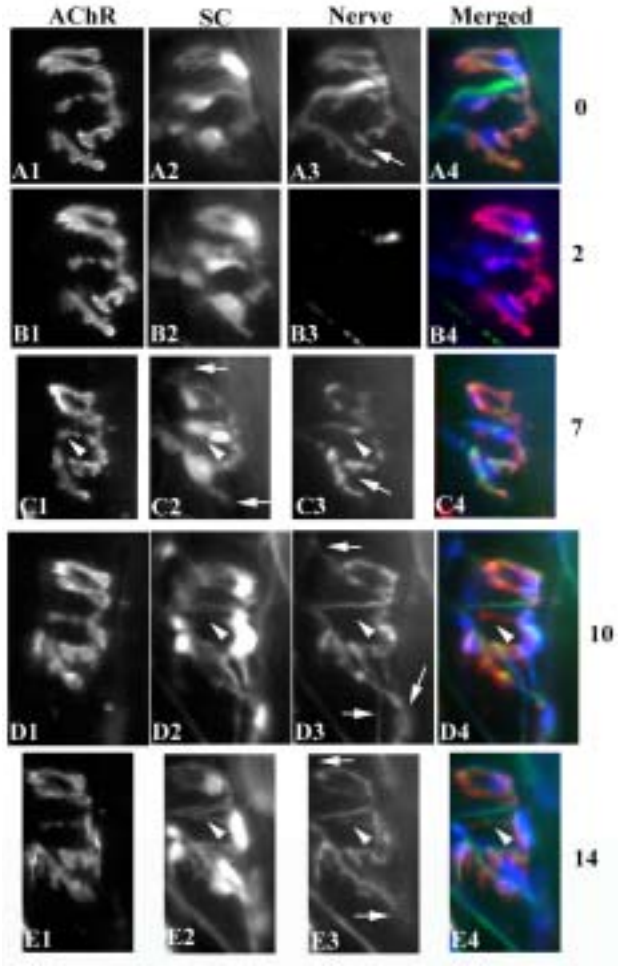

Figure 2.11: Regenerating axons re-occupy portions of AChRs following withdrawal of escaped fibers

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves were viewed repeatedly at one endplate over the course of 14 days following nerve crush. In the merged images (A4, B4, C4, D4 and E4), AChRs, SCs and nerves appear as blue, red and green, respectively. When the first images were collected before nerve crush, an axon wrapped by Schwann cells entered into the endplate from the left of panel A3 and divided into two branches that covered all the AChRs (Panel A). When the second images were taken two days after nerve crush, the nerve terminals had degenerated (B3). On day7, it appears that the regenerating axon

began to reinnervate the endplate following Schwann cell processes but with an altered branching pattern from day 0 (compare arrows in A3 and C3). In A3 the lower nerve branch innervated only the lower synaptic gutter but in C3 the lower branch had branched over a SC connection between gutters to innervate a portion of an upper gutter as well. Furthermore, at day 7, the regenerating axon did not cover a portion of AChRs which were occupied by two Schwann cell bodies (arrowheads in C1, C2 and C3). Note that two small Schwann cells processes extended out of the endplate (arrows in C2). On day 10, at least three escaped fibers extended out of the endplate and two of them appeared to follow the Schwann cell processes growing before day 7 (compare arrows in C2 and D3). Note these two SC processes had became longer and bigger than on day 7. A portion of AChRs were still not covered by the axon (arrowheads in D3 and D4), and two Schwann cell bodies moved to the side of the endplate but left a process covering the portion of AChRs (compare arrowheads in C2 and D2). When the last images were taken on day 14, the portion of AChRs had been re-occupied by the axons and the SC processes (arrowheads in E2, E3 and E4) and at least two escaped fibers had withdrawn (compare arrows in D3 and E3).

Escaped fibers grow to innervate adjacent endplates by following Schwann cell processes.

During nerve regeneration, many axons that grow back to endplates by following old Schwann cell tubes form escaped fibers that grow beyond the endplate (Son and Thompson, 1995; O'Malley et al., 1999; and see above). In many cases, these escaped fibers apparently come to reinnervate adjacent endplates. These endplates thus become innervated by an axon arriving from a new direction. Here, we refer to such inter-endplate axons formed by escaped fibers as "escaped fiber bridges". As reported previously from static observations in rats (Son and Thompson, 1995; Love and Thompson, 1999; Love and Thompson, 2003), we observed that these escaped fiber bridges were always associated with Schwann cell processes that, because they interconnect two endplates, are called "Schwann cell bridges" (Love and Thompson, 1999; Love and Thompson, 2003). To understand the temporal relationship between escaped fiber bridges and Schwann cell bridges, we imaged AChRs, Schwann cells and axons repeatedly over the course of nerve regeneration. We focused our attention on endplates with Schwann cell extensions that looked as if they might form a bridge with an adjacent endplate. In many cases, we found that an escaped fiber bridge and its associated Schwann cell bridge formed between two images collected a day apart. In these cases we were therefore unable to discern whether the escaped fiber bridge followed or preceded the Schwann cell bridge. However in 9 soleus muscles we were able to observe the formation of 18 escaped fiber bridges. In each of these 18 cases, the escaped fiber bridge grew over a

pre-existing Schwann cell bridge (Fig. 2.12). In most cases (14 of the 18 cases) the escaped fiber bridge reinnervated a denervated endplate site, as this axon arrived in advance of any axon entering the endplate through the old Schwann cell tube (Fig. 2.12). In 4 of the 18 cases, the endplate became reinnervated by the escaped fiber bridge even though the endplate had been reinnervated shortly before by an axon regenerating through the old Schwann cell tube for this endplate. Even endplates reinnervated initially by an escaped fiber bridge on occasion became reinnervated by a second axon regenerating through the old Schwann cell tube. This was the case for 3 of the 14 endplates discussed above. Thus, in 7 of these 18 cases, the endplate became polyneuronally innervated. We followed 6 of these cases of polyneuronal innervation for a sufficient period of time to observe the loss of this polyneuronal innervation. In 5 of the cases the loss was by the escaped fiber bridge (Fig. 2.13); in 1 of the cases the loss was by the axon arriving over the old Schwann cell tube (Fig. 2.12 Panel E and F). In cases where the loss was by the escaped fiber bridge, the Schwann cell bridges remained for a short time following withdrawal of the escaped fiber and then disappeared. In a few additional cases, we extended our observations to view endplates for even longer periods following nerve regeneration (20 to 35 days) in order to study the stability of Schwann cell bridges and the innervation by escaped fiber bridges. In 6 cases where it appeared the endplates had either been reinnervated exclusively by an escaped fiber bridge or in the 1 case where the escaped fiber bridge appeared to have survived competition with a second axon reinnervating the endplate through the old Schwann cell tube, the escaped fiber

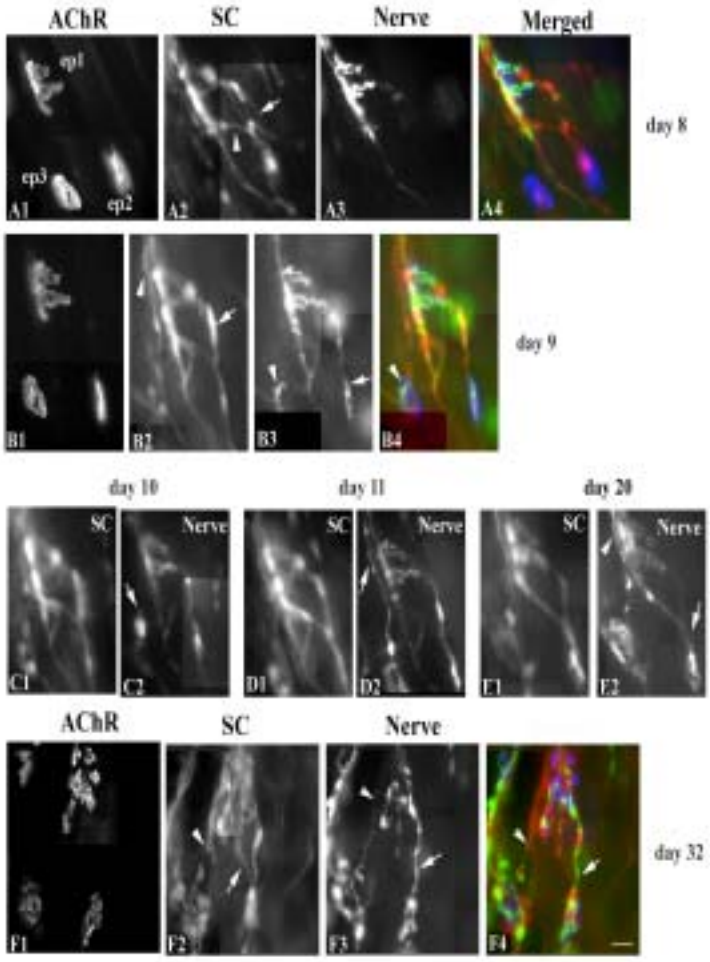

Figure 2.12: Escaped fibers can generate polyneuronal innervation when they reinnervate denervated endplates by following Schwann cell processes or old Schwann cell tubes

Acetylcholine receptors (AChR), Schwann cells (SCs), and nerves at three endplates (ep1, ep2, ep3) were viewed repeatedly over the course of 32 days following nerve crush. In the merged images (A4, B4 and F4), AChR, SCs and nerves appear as blue, red, and green, respectively. New bungarotoxin was applied on days 8, 20 and 32. At the time the first images were taken on day 8, ep1 has been reinnervated, but ep2 and 3 remain denervated (A3 and A1). SC

processes appear to connect ep2 to ep1 (arrow and arrowhead in A2), forming a SC bridge. By day 9 an escaped fiber has grown from ep1 along the SC bridge to reinnervate ep2. Also by day 9 ep3 has been reinnervated by a sprout entering from the left of the picture (arrowhead in B3). Images on days 10, 11, and 20 suggest that the axon reinnervating ep3 grows (arrow in C2, D2) back up the old SC tube (arrowhead in B2). By day 20, this axon appears to branch, enter the old SC tube leading to ep2 (arrowhead in E2), and reinnervate ep2 (arrow in E2). Thus on day 20 ep2 is polyneuronally innervated by an axon that grew from an adjacent endplate along old SC tubes and by an escaped fiber that arrived over a SC bridge. On day 32, the animal was sacrificed and these endplates located in vitro (F1-F4). By this time, ep2 is innervated only by the escaped fiber from ep1 (arrow in F3) and the old SC tube leading to ep2 has lost its axon (arrow in F2, compare with F3). However the axon growing back up the old SC tube from ep 3 (arrowhead in F2) is still present (arrowhead in F3). Bar 20 μm

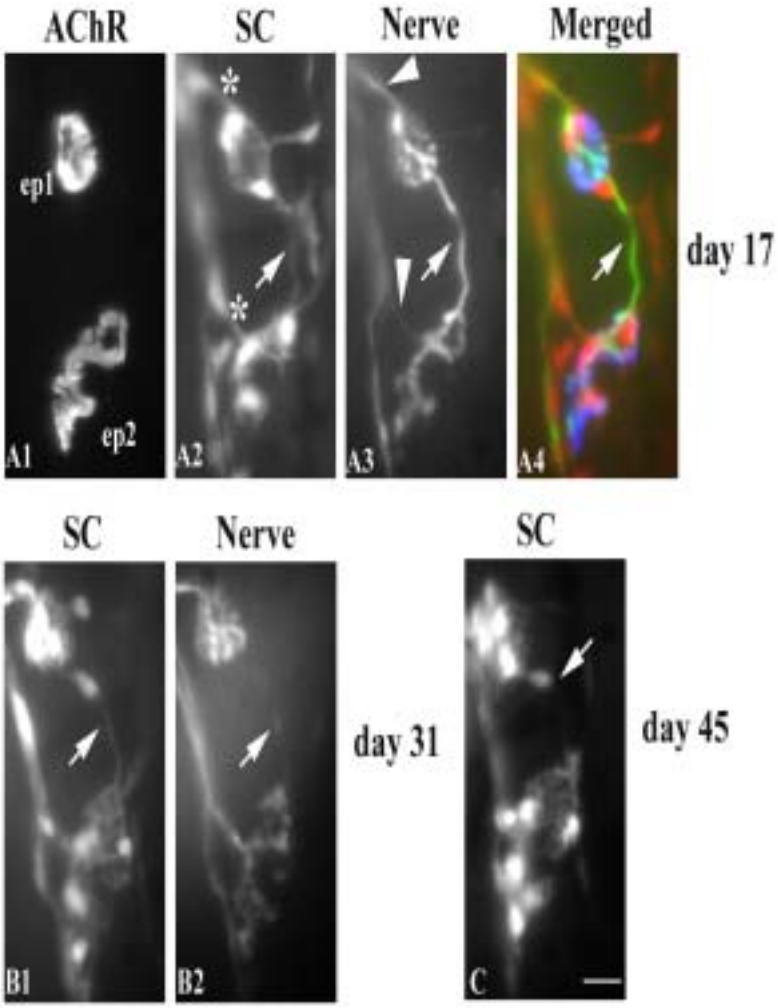

Figure 2.13: Polyneuronal innervation turn to single innervation by withdrawal of terminal sprouts, and Schwann cell bridges withdraw consequently.

Acetylcholine receptors (AChR), Schwann cells (SCs), and nerves at two endplates (ep1, ep2) were viewed repeatedly over the course of 45 days following nerve crush. In the merged image (A4), AChR, SCs and nerves appear as blue, red, and green, respectively. New bungarotoxin was applied on days 17, 31 and 45. On day 17 following nerve crush, a terminal sprout has interconnected ep1

and ep2 associated with a SC bridge (arrow in A2, A3 and A4). Note ep1 and ep2 has been reinnervated by axons (arrowheads in A3) upon two old SC tubes (asterisks in A2). On day 31, the terminal sprout has withdrawn (arrow in B2) but the SC bridge is present (arrow in B1). Thus, polyneuronal innervation formed prior to day 17 on ep1 become single innervation (compare panel A and B). On day 45, the SC bridge has dissappeared (arrow in C). Bar 20 μm

bridges persisted. Thus, it appears that endplates can be stably reinnervated by escaped fibers.

In some cases, we found two escaped fibers present within one Schwann cell bridge or old Schwann cell tube (Fig. 2.14 Panel A-B). Moreover, the escaped fibers were found growing in opposite directions along one Schwann cell bridge. Thus, it appears that Schwann cell bridges are capable of supporting growth in either direction between the endplates linked by such a bridge.

Escaped fibers that innervate nearby endplates commonly grow in a retrograde direction along old Schwann cell tubes.

In at least 6 of 14 cases where we observed formation of an escaped fiber bridge, we were able to observe that the escaped fiber continued to grow in a retrograde direction in the old Schwann cell tube. In many cases we could observe the growing tip of the escaped fiber entering the old Schwann cell tube. This type of growth made it appear that some endplates innervated by escaped fibers were polyneuronally innervated, by the escaped fiber and an axon entering through the old Schwann cell tube. In 3 cases, we were able to follow this retrograde growth for a sufficient length of nerve to observe that the axon branched at a branch point in the intramuscular nerve and then grew in the orthograde direction down this old Schwann cell tube to innervate an adjacent endplate (Fig2.15 Panel A-C). In a number of cases we were able to follow such retrograde reinnervation for several weeks (some as long as 30 days). In these cases this retrograde innervation was stable. In many other cases, we observed a retrograde axon leaving an endplate innervated by an escaped fiber bridge, but in

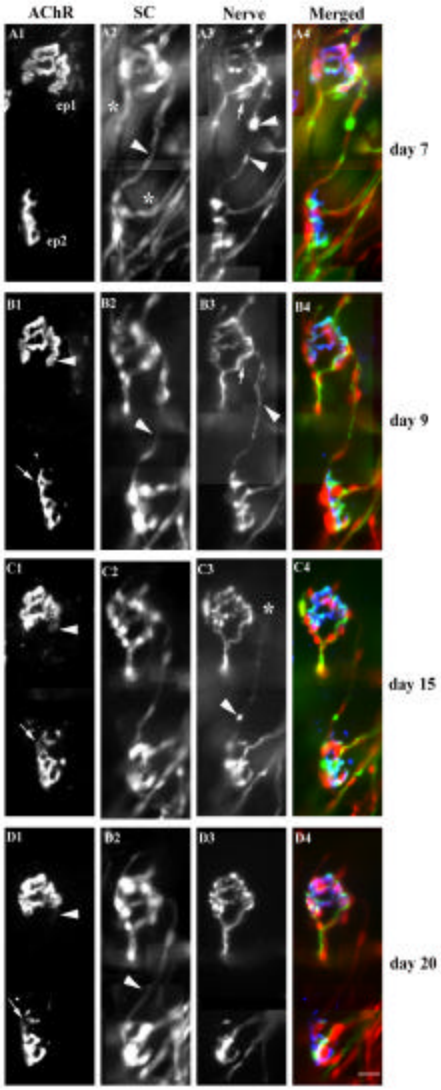

Figure 2.14: Some loss of AChR sites is related to withdrawal of terminal sprouts and the loss of polyneuronal innervation.

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at two endplates (ep1 and ep2) were viewed repeatedly during the course of reinnervation for 20 days following nerve crush. In the merged images (A4, B4,

C4, and D4), AChRs, SCs, and nerves appear as blue, red, and green respectively. New rhodamine-bungarotoxin was applied on days 7, 15, and 20. When the first images were collected on day 7 (Panels A1-A4), both ep1 and ep2 had been reinnervated by axons arriving over old SC tubes (asterisks in A2). A number of escaped fibers associated with SC processes leave the top of ep1 (A2 and A3) and at least one leaves the bottom of ep2. In addition there is a SC bridge (arrowhead in A2) that has connected ep1 and ep2, and two escaped fibers have grown towards each other along this bridge (arrowheads in A3). Two days later, on day 9, there is a continuous nerve labeling (arrowhead in B3) along this bridge connecting ep1 and ep2 (arrowhead in B2). It is not clear whether there are two axonal processes on this bridge at this time, but a later image (see below) suggests, at a minimum an axon from ep1 has reached ep2. On day 15, an axon with a bulbous end that is continuous with ep1 is apparently withdrawing from the top of ep2 (arrowhead in C3). By the time of the image on day 20, this axon has disappeared, despite the continued presence of the SC bridge (arrowhead in D2). Comparison of the AChR staining on days 9-20, suggests that the receptors at the top of ep2 are becoming progressively weaker in intensity, consistent with loss of receptors accompanying the loss the axon from ep1 (arrow in D1). These images also show a second type of loss of AChR sites. Comparisons of the AChR in ep1 between days 7 and 20 show a loss of intensity and eventual disappearance of receptors in the bottom left portion of this endplate (compare arrowheads in B1, C1, and D1). At day 7 nerve appears above this receptor area (arrow in A3), but by day 9 this area appears devoid of nerve (arrow in B3), despite the nearby presence of a branch of the axon that has regenerated up the old SC tube. Thus loss of receptor areas is correlated with the disappearance of innervation of these areas. The images collected at day 7 suggest that this area was initially innervated by the axon reinnervating the endplate through the old SC tube. The loss is correlated with the growth of the escaped fiber (arrowhead in B3) to ep2. Bar 20 μm

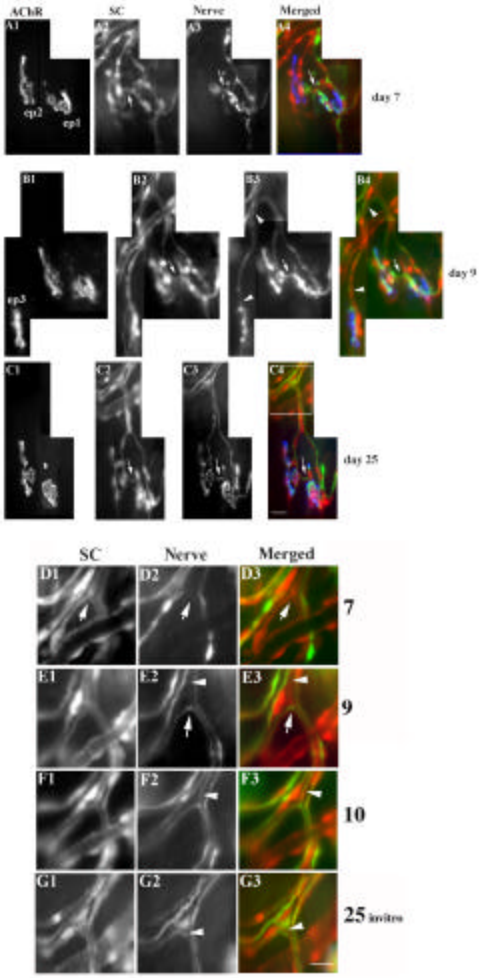

Figure 2.15: Escaped fibers that innervate an adjacent endplate by growing over Schwann cell bridge often continue to grow in the retrograde direction over old Schwann cell tubes.

Acetylcholine receptors (AChR), Schwann cells (SCs) and nerves were viewed repeatedly over the course of 25 days following nerve crush. In the merged images (A4, B4, C4, D4, E4, F4, and G4), AChRs, SCs, and nerves appear blue, red, and green, respectively. New bungarotoxin was applied on day 7 and 25. At the time the first images were collected on day 7, ep1 has become reinnervated by

an axon arriving over the old SC tube. A SC bridge (arrow in A2) has formed between ep1 and the adjacent, uninnervated ep2; escaped fibers are growing from ep1, one of which is growing on this SC bridge (arrow in A3). By day 9 the escaped fiber (arrow in B3) has not only reinnervated ep2, it has grown back up the old SC tube and then down the old SC tube leading to an adjacent endplate (ep3) not shown in the previous images. This escaped fiber persists when this area of the muscle was examined in vitro on day 25. The images D1-G3, show enlargements of the boxed area in C4 that were obtained in the images collected on day 7, 9, 10, and 25. These images show a bifurcation in the SC tube leading to ep2 and ep3 (arrow in D1) at day 7 at which time there is no axon present (arrow in D2), the growth of an axon back up the tube, entering the tube to ep3 (arrow in E2), and extension of a small retrograde process towards the CNS (arrowhead in E2). The latter, retrograde process is thicker on day 10 (arrowheads in F2 and F3), but by day 25 is gone (arrowheads in G2 and G3). Images such as these show the very complex axonal branching that can occur upon reinnervation of a denervated muscle, branching that is owed to the pathways provided by SCs. Bar 20 μm

these cases, we were unable to follow this axon for any distance and discern whether it had or had not been successful in reinnervating an additional endplate. Some of these axons were persistent and some of them were later withdrawn (Fig. 2.15 Panel D-G). The generation of these retrograde axons considerably complicates determination of how individual endplates have become reinnervated and provide additional examples of how Schwann cell guidance alters the pattern of innervation of muscles during reinnervation.

Other cases of AChRs loss and endplate remodeling following nerve regeneration

In addition to the loss of AChR sites during reinnervation reported above that were apparently due to failure of reoccupation of sites by the regenerating nerve, we also found cases of receptor loss that clearly had other explanations.

At 16 endplates in 11 soleus muscles reinnervated following nerve crush we observed receptor loss in which we had also obtained images both early in reinnervation and at long times (> 15 days) following reinnervation. At 5 of these endplates, nerve terminals were initially present over receptor sites but were subsequently withdrawn (Fig. 2.16). These receptor sites were lost over the course of the subsequent ca. 20 days. We could not discern the presence of more than one axon in each of these 5 cases, although we cannot exclude the possibility that two axons entered the endplate through the old Schwann cell tube but could not be resolved in our images (we provide estimates of the extent of polyneuronal innervation below).

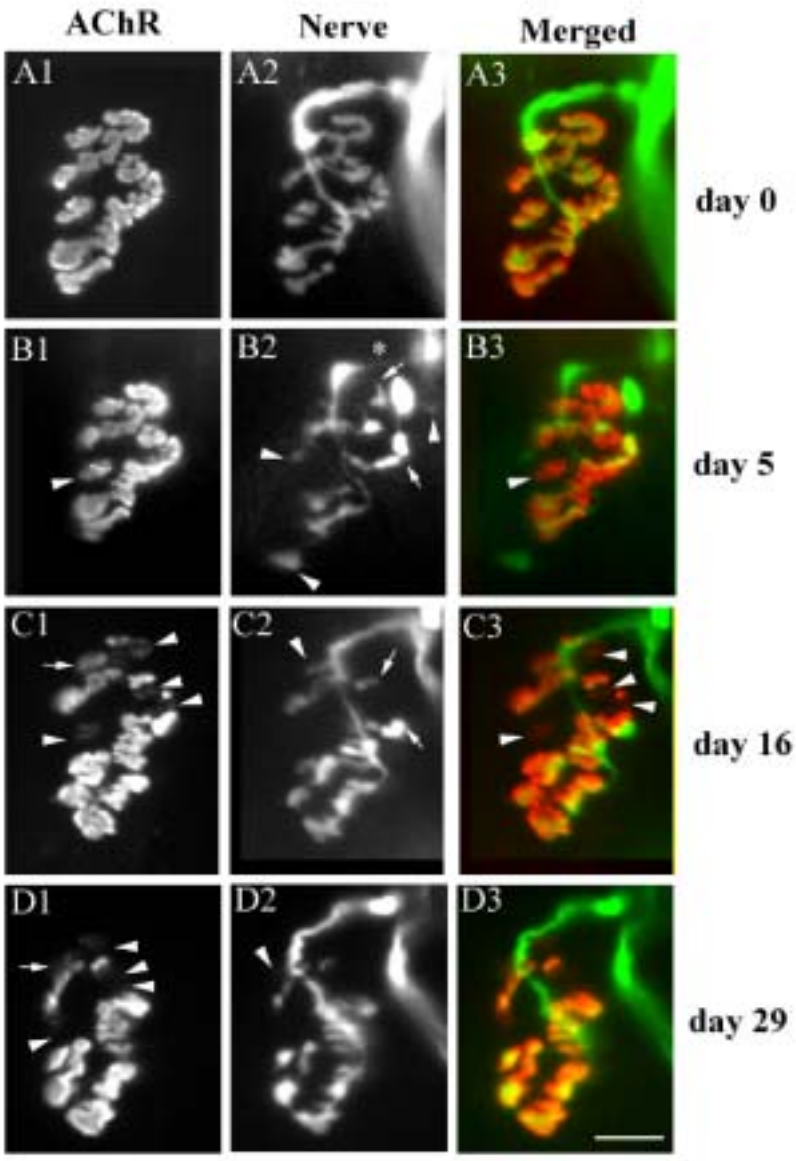

Figure 2.16: Failure of reoccupation of acetylcholine receptor areas during reinnervation and withdrawal of innervation from sites initially reinnervated leads to loss of synaptic areas

Acetylcholine receptors (AChR), Schwann cells (SCs), and nerves were viewed prior to denervation and then over the course of 29 days of reinnervation. Only

AChR and nerve images are shown here. In the merged images (A4, B4, C4, D4), AChR and nerves have been colored red and green, respectively. New rhodamine-bungarotoxin was added on days 0, 16, and 29. The first images were collected on day 0, immediately prior to nerve crush (A1-A3). A single axon enters the endplate and divides into 3 branches that cover all the acetylcholine receptors. When the next image was collected on day 5, this endplate was already reinnervated by an axon arriving over the old SC tube (asterisk in B2), but the reinnervating axon did not faithfully re-occupy the AChR sites. First of all, there are at least 3 escaped fibers (arrowheads in B2) that extend beyond the AChRs. Secondly, there are also AChR sites (arrowheads in B1 and B3) to which the nerve did not apparently regenerate or establish stable occupation. In the images on day 16 there are some nerve processes that have disappeared that previously occupied synaptic sites at day 5 (compare the arrows in B2 and C2). Moreover the corresponding receptor sites, as well as the receptor site not occupied on day 5, have lost receptor labeling (arrowheads in C1 and C3). The faintly labeled receptor sites have disappeared by day 29 (arrowheads in D1). On day 29, the nerve terminal pattern is not much changed from day 16, except for the disappearance of one terminal branch indicated by the arrowheads in C2 and D2. Note that the receptor areas for this site are correspondingly faint (arrow in D1). Images of SCs are not included in this figure because of faintness of SC labeling on day 5. Bar 20 μm

The observations above suggest that some of the loss of AChR sites at reinnervated endplates occurs as a consequence of failure of nerves to reoccupy some endplate areas. In 2 soleus muscles we observed 10 endplates before and after nerve crush, and found 2 endplates that had a portion of their AChR sites unoccupied by nerve approximately 13 days following the beginning of reinnervation. We then denervated the muscle again by resecting the nerve. We found that the sites not reoccupied following nerve crush that would have been expected to be subsequently lost, persisted without loss for another at least 35 days following nerve resection (Fig. 2 17). These observations suggest, not surprisingly given the results of Balice-Gordon and Lichtman (1994), that the loss of these receptor sites requires the disparity in activation of occupied and unoccupied receptor sites by the nerve.

One of these endplates was polyneuronally innervated in early images by an escaped fiber bridge and by an axon that had regenerated through the old Schwann cell tube (Fig. 2.14). Subsequently, after the escaped fiber bridge disappeared, receptor areas were observed that lacked Schwann cell and axon coverage, and this area subsequently disappeared.

At 6 of these endplates we found loss of receptor areas at sites where an escaped fiber bridge left one endplate (Fig 2.18). In none of these cases could more than one axon be detected, and the association of the loss with the escaped fiber suggests mechanisms in operation that are independent of inter-neuronal competition and similar to those observed cases where endplates lost receptor sites at sites of nerve entry (see above).

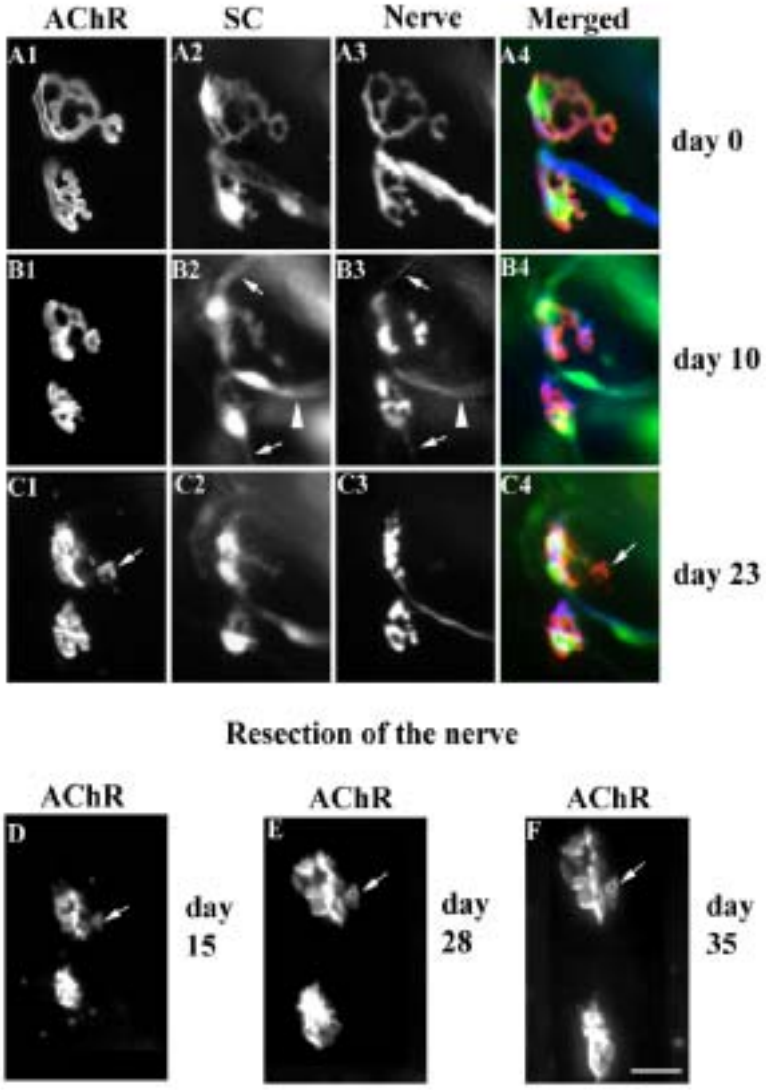

Figure 2.17: Unoccupied synaptic sites do not lose receptors if the muscle fiber is denervated.

Acetylcholine receptors (AChR), Schwann cells (SCs) and nerves at an endplate were viewed repeatedly during reinnervation over the course of 23 days following nerve crush and for a further 35 days following resection of the nerve. AChRs, SCs and nerves appear as red, blue and green respectively in the merged images (A4, B4, C4). New bungarotoxin was applied before each observation. The first

images were collected on day 0, i.e. before nerve crush. Nerve terminal branches (A3) cover each of the AChR sites (A1). At 10 days following nerve crush, the endplate has been reinnervated by an axon (arrowhead in B3) entering over the old SC tube (arrowhead in B2). The nerve extends two escaped fibers from the top and bottom of the endplate (arrows in B3) associated with two SC processes (arrows in B2). By day 23, the escaped fibers and SC processes have been withdrawn, but additionally nerve processes but not SC processes are now missing from an AChR site previously occupied on day 10 (arrows in C1 and C4). From other observations reported here, one would expect this AChR site to be lost over subsequent views. To determine if innervation of the rest of the endplate was important for this loss, the nerve to the muscle was resected following the observation on day 23. This same endplate was then viewed 15, 28, and 35 days later; the last observation was after sacrifice of the animal and fixation of the muscle. The previously unoccupied synaptic site expected to lose receptors (arrow in D, E, F) was stable over the 35 days of denervation. The images in E and F are shown are higher magnification than those in the upper panels. Bar 20 μm

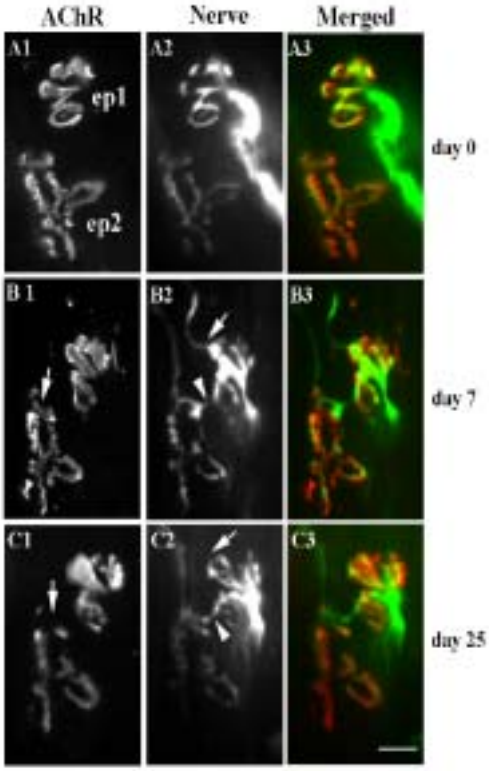

Figure 2.18: Loss of AChRs occurs at sites where an escaped fiber bridge left one endplate

Acetylcholine receptors (AChRs), Schwann cells (not shown) and nerves at two endplates (ep1 and ep2) were viewed repeatedly over the course of 25 days following nerve crush in a soleus muscle. In the merged images (A3, B3 and C3), AChRs and nerves appear as red and green, respectively. New rhodamine-bungarotoxin was applied on day 0, 7 and 25. On day 0: before nerve crush, ep1 and ep2 were innervated by two axons respectively. However, 7 days following nerve crush, ep1 was only reinnervated by an escaped fiber from ep2 (arrowhead in B2) and it extended out of ep1 as a new escaped fiber (arrow in B2). On day 25, the escaped fiber bridge was persistent and the new escaped fiber had withdrawn already (compare arrow in B2 and C2). Furthermore, loss of AChR occurred in several sites on ep2 and especially in which the escaped fiber left ep1 (compare arrows in B1 and C1). Bar 20 µm

In the 4 remaining cases there was loss of AChR sites, but CFP labeled nerve remained above the site where receptors were previously present (Fig 2.14 Panel B-D). It is possible that this portion of the regenerating nerve had ceased to function as a terminal or had always been a non-terminal branch.

A few endplates sites gain AChR sites following nerve regeneration

In addition to loss of AChR receptor sites, we also observed a few instances where new AChR sites were added. These added AChR sites were always associated with Schwann cell processes and nerves that had escaped endplates during reinnervation. In some cases, we could observe that Schwann cell processes and escaped fibers preceded the addition of AChR. Moreover, in some cases, we found that AChR addition occurred along the escaped fibers and the endplates changed dramatically because of AChR loss occurring at the same time (Fig. 2.19).

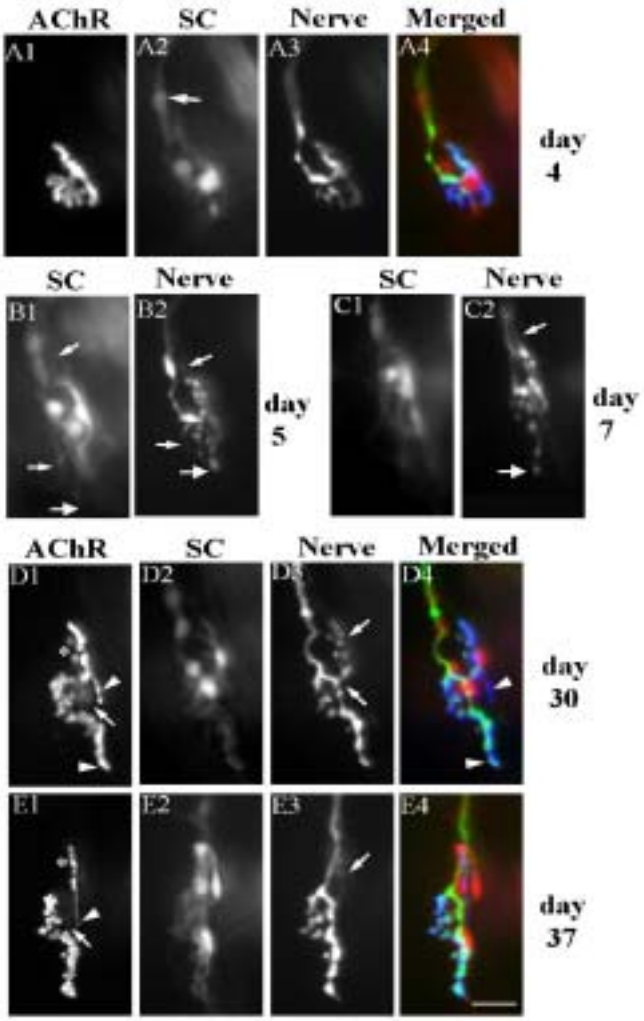

Figure 2.19: In some cases addition of new AChR sites occurs along the pathway of escaped fibers, and when combined with loss of receptor sites, the configuration of the synaptic site is dramatically altered.

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at one endplate were viewed repeatedly over the course of reinnervation for 37 days following nerve crush. In the merged images (A4, D4 and E4), AChRs, SCs and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day 4, 30 and 37. The first images were collected 4 days

following nerve crush and the endplate was already reinnervated by a nerve arriving over the old SC tube (arrow in A2). Just one day later, on day 5, the SCs had extended at least three SC processes (arrows in B1) over which three escaped fibers had grown (arrows in B2). Note that all three SC processes are longer than their associated escaped fibers (compare arrows in B1 and B2). On day 7, the top and bottom escaped fibers had become thicker but the one indicated by the central arrow in B2 has withdrawn (arrow in C2). No changes in AChR were observed from day 4 to 7 (data not shown). On day 30, however, such changes were noted. First, new AChR sites (lower arrowhead in D1) have been added along the course of the lower escaped fiber. Note that the top escaped fiber (upper arrow in B2) has withdrawn. Second, some AChR sites within the old synaptic site are now faintly labeled (compare arrow in D1 and bottom arrow in D3). When observed in vitro at day 37 after sacrifice of the animal, these sites appear to be dimmer still. In addition one AChR area is not occupied by the nerve terminal (arrowhead in D1 and D4). Further on day 30, one AChR site was not occupied by a nerve terminal (upper arrowhead in D1 and D4). On day 37, this receptor site has become smaller and more faintly labeled (compare arrowhead in D1 and E1). One last change between days 30 and 37 is that some nerve branches initially present have withdrawn (compare top arrow in D3 and arrow in E3) and their corresponding AChR sites have been lost (compare asterisk in D1 and E1). These changes make the AChRs present early in reinnervation (A1) strikingly different from those present a month later (E1). Bar 20 μm

Estimates of the incidence of different types of reinnervation and of polyneuronal innervation

The observations reported above give frequencies of occurrences of various types of innervation and outcomes in special circumstances. Because of our desire to minimize exposure of muscles to illumination, observations were commonly limited to only a few endplates per muscle and commonly these endplates were chosen for special reasons, e.g. innervation by an escaped fiber, AChR areas not covered by axons or Schwann cells. To obtain a set of observations where the incidence of some of the features we report above would be more representative of the events overall during reinnervation, we denervated 4 soleus muscles in CFP/GFP transgenic animals by crush of the soleus nerve, sacrificed the animals 9 days later, and observed their muscles in vitro. We observed 103 endplates that could be viewed en face. Approximately 2/3 of these fibers appeared to be singly innervated as only one axon appeared to enter the endplate either through the old Schwann cell tube or as an escaped fiber (in this case the escaped fiber did not enter the old Schwann cell tube). The remaining 1/3 of the fibers were potentially polyneuronally innervated. However, this is probably an overestimate of the extent of polyneuronal innervation. More than half of these potential examples of polyneuronal innervation were cases where an escaped fiber bridge linked two endplates that also had an axon in their old Schwann cell tube. While this would imply that it is possible that one or both of these endplates were reinnervated by axons arriving both over the old Schwann cell tube and over the Schwann cell bridge, we have shown above that some of these cases can be accounted for by a single axon that innervates one endplate,

crosses to an adjacent endplate along a Schwann cell bridge, and then grows retrogradely in the old Schwann cell tube of the second endplate. There were 14 cases where polyneuronal innervation appeared to arise from more than one axon entering the endplate; however, in half of these 14 cases we could see that some of these multiple axons arose as a consequence of branching in the old Schwann cell tube. Given these considerations, we believe the extent of polyneuronal innervation is probably less than 25% at this time. However, the incidence of polyneuronal innervation in muscles following crush could be more or less than 25%, since the results shown here were from static observations en face. These endplates also allowed us to estimate the number of escaped fiber bridges that form in muscles during reinnervation. Eighteen of the 103 endplates had such bridges and in an additional 9 cases there was an escaped fiber that projected out of the focus and yet perhaps reached another endplate. (see table 2.2)

Table 2.2: Summary of data Estimates of the incidence of different types of reinnervation and of polyneuronal innervation

<table>
<tr><td rowspan="2">Endplates reinnervated by single axons</td><td rowspan="2">66 (64.7%)</td><td colspan="2">Endplates reinnervated by axons from old SC tubes
62</td></tr>
<tr><td colspan="2">Endplates reinnervated by escaped fibers
4</td></tr>
<tr><td rowspan="4">Endplates reinnervated by more than one axons</td><td rowspan="4">36 (35.3%)</td><td rowspan="2">Endplates reinnervated by axons from old SC tubes

15</td><td>Endplates reinnervated by more than one axons from old SC tubes
7</td></tr>
<tr><td>Endplates reinnervated by two branches possible from same axon
8</td></tr>
<tr><td rowspan="2">Endplates reinnervated by axons from two directions

21</td><td>Endplates reinnervated by an axon and an escaped fiber which is out of focus
9</td></tr>
<tr><td>Endplates reinnervated by an axon and escaped fiber</td></tr>
</table>

DISCUSSION

We believe our results show the roles Schwann cells play in nerve regeneration. Repeated vital imaging of muscles of transgenic animals allows us to observe junctions before, during and after nerve regeneration. Schwann cells extend processes in response to denervation and these processes become the pathways for escaped fibers during nerve regeneration. Meanwhile, a portion of AChRs at individual endplates is not occupied by Schwann cells and their processes after denervation, and then regenerating axons following Schwann cell processes will not reoccupy these receptors. These receptors may be subsequently lost after nerve regeneration. In some cases, a Schwann cell process interconnecting two endplates, guides an escaped fiber to innervate adjacent endplates. When a second axon arrives over the old Schwann cell tube leading to this endplate, polyneuronal innervation is subsequently formed. In some cases, these escaped fibers continue to grow over old Schwann cell tubes retrogradely and even innervate endplates on other sides of these tubes. Since Schwann cell bridges and old Schwann cell tubes guide escaped fiber innervation, Schwann cell activities are crucial for nerve reinnervation and may change the whole reinnervation pattern in a certain area. Our results also show that AChR loss is related to withdrawal of regenerating nerve terminals, extending persistent escaped fibers and loss of polyneuronal innervation. Further observations show the loss of receptors is due to competition between active and inactive receptors long-term following nerve regeneration.

Short Schwann cell extensions in control muscles

We believe that our repeated observations of normal muscles suggest the following points. First, in most of our control experiments, we did not find changes of AChRs, Schwann cells and nerves over the course of vital imaging. These results suggest AChRs, Schwann cells and nerves are morphologically stable in muscles of young adult mice, consistent with previous observations (Rich and Lichtman, 1987; O'Malley et al., 1999; Wigston, 1989). Moreover, AChRs, nerves and Schwann cells can be repeatedly imaged without effects from imaging and surgery. Second, we found that about 50% of the endplates had short Schwann cell extensions, in agreement with observations in frog muscle preparations and EM micrographs of mouse muscles (Robbins, Polak, 1988; Macleod et al., 2001). Rochon et al. (2001) showed nerve activities modulate Ca^{2+} activities in terminal Schwann cells in mice. On the other hand, Schwann cells also can modulate transmitter releasing at frog endplates (Robitalle 1998). Our results show that Schwann cells extend and retract short processes in a dynamic fashion in the normal muscle, which may be correlated with Ca^{2+} activities inside of terminal Schwann cells modulated by nerves. These observations may be direct evidence suggesting that Schwann cells function actively in maintenance of normal junctions. Moreover, these dynamic Schwann cell extensions also suggest there may be some factors which hold a dynamic equilibrium between extension and withdrawal of these short Schwann cell processes, and this equilibrium may be sensitive to synaptic activities. For example, when the equilibrium is broken by denervation, Schwann cell processes will extend relatively long processes.

Finally, these short Schwann cell extensions without nerve sprouts accompanying them also suggest nerve sprouting might require persistent Schwann cell processes.

Terminal Schwann cells extend processes in response to muscle denervation and these processes guide the formation of escaped fibers

Previous observations showed terminal Schwann cell processes sense changes of the neuronal activity at NMJs and extend processes in response to denervation and paralysis (Son and Thompson, 1995a; O'Malley, 1999). In agreement with previous observations, our results show that terminal Schwann cells extend their processes and these processes migrate along muscle fibers following denervation in most cases. Moreover, we found that not all endplates extend Schwann cell processes, and some of these Schwann cell processes are dynamic. This suggests that the signals that determine process extension are generated locally. The process extension following denervation may have nothing to do with muscle fiber types, since we found these phenomena in soleus muscles with 99% slow muscle fibers as well as in sternomastoid muscles with fast fibers. In agreement with previous observations (Son and Thompson, 1995a; O'Malley, 1999), our observations also show that regenerating axons grow over old Schwann cell tubes and continue to grow over preexisting Schwann cell pathways. These pathways are formed by terminal Schwann cell processes extending in response to nerve crush. Further, during nerve regeneration, the axons arriving over old Schwann cell tubes or Schwann cell bridges to innervate old synaptic sites (see below), exactly follow the Schwann cell processes even not

covering AChRs. Thus, Schwann cell process pathways are substrates for regenerating axons to grow not only in old Schwann cell tubes and TSC processes beyond endplates areas, but also inside endplates as well.

Schwann cell bridges guide escaped fibers to innervate adjacent endplates.

Following nerve regeneration, some endplates are innervated by more than one axon. This is called polyneuronal innervation (Rich and Lichtman 1989). In agreement with previous observations (Son and Thompson, 1995 a), we believe that our results from vital imaging also explain how polyneuronal innervation forms. Escaped fibers grow over Schwann cell processes interconnecting two endplates to innervate adjacent endplates. Polyneuronal innervation is formed at these endplates if there is additional reinnervation by axons growing over old Schwann cell tubes. We also observed that escaped fibers innervating adjacent endplates along Schwann cell bridges continued to grow over the old Schwann cell tubes retrogradely. Therefore, in some cases, it is hard to determine whether an endplate is polyneuronally innervated or singly innervated. Since one escaped fiber can innervate several endplates due to the guidance of Schwann cell bridges and then old Schwann cell tubes, we believe that Schwann cells are able to change the innervation pattern in each of the muscle.

It seems that there is no certain direction for the growth of escaped fibers over Schwann cell bridges or old Schwann cell tubes. Our observations suggest that axons only try to cover more Schwann cell processes during nerve regeneration. Furthermore, during retraction of escaped fibers and the related

Schwann cell processes, escaped fibers always withdraw faster than Schwann cell processes. In some cases, Schwann cell processes stay while escaped fibers disappeared. These observations might suggest Schwann cells might not participate in escaped fiber retraction. However, these persistent Schwann cell processes appear unattractive to nerves in contrast to their status during earlier nerve regeneration. This is especially obvious in cases where terminal sprouts withdraw but leave Schwann cell bridges for a very long time, and these Schwann cell bridges do not induce and guide terminal sprouts any more. This suggests that the contact between Schwann cells and nerves can determine the properties of Schwann cells, and therefore change interactions between Schwann cells and regenerating axons, such as Schwann cell guidance and induction of regenerating axons. This kind of change appears to be important for synapse restoration by making axons reinnervate more synaptic sites other than randomly growing into perijunctional space.

AChR loss following nerve regeneration

Previous researchers reported loss of polyneuronal innervation which was preceded and then followed by AChRs loss (Rich and Lichtman, 1989, Culican SM et 1998). In our experiments, we found one case where the polyneuronally innervated endplate lost its receptors following an escaped fiber withdrawal as well as some other cases that might be explained by loss of polyneuronal innervation. Additionally, our observations show the loss of polyneuronal innervation is not the only reason that endplates lose their receptors following

nerve regeneration. Our results suggest that the loss of some AChR is a consequence of lack of occupation of AChR sites by Schwann cells. Not only do Schwann cells extend processes in response to denervation, but also leave some AChR areas unoccupied before nerve regeneration. Unlike the coverage by Schwann cell processes and nerve terminals in AChRs at endplates of normal muscles, Schwann cells lose coverage of AChRs gradually following denervation. Since regenerating axons follow the Schwann cell processes inside of endplates, these regenerating axons ignore some AChRs that are not covered by Schwann cell processes. Subsequent observations in some of these cases show that the AChRs unoccupied by Schwann cell processes and regenerating axons are lost.

In both wild type and transgenic mice, we found that about half of the endplates we observed lost more than 5% of their AChRs. However, more than 60% endplates had lost some Schwann cell coverage following denervation. This then can not account for AChRs loss at about 10% endplates. We found in some cases that growth of Schwann cell processes inside of endplates appeared to increase during nerve regeneration, suggesting that regenerating axons may stimulate the growth of Schwann cell process inside individual endplates. Possibly, AChRs that are reoccupied by Schwann cell processes and regenerating axons will avoid elimination.

In some cases, axons that previously covered a portion of AChRs at individual endplates withdraw and leave these receptors unoccupied and the unoccupied AChRs are then gradually lost. We do not have further evidence for why axon withdrawal follows nerve regeneration in these cases, but these

observations could be explained by changes inside endplates that we may not be able to observe morphologically. Based on the observations above, we believe that reinnervation by a single axon is able to induce AChR loss following nerve regeneration.

Moreover, AChR loss was also found at sites where endplates extended escaped fiber bridges and these sites seem to become pre-terminal axons without synaptic vesicles. Previous observations from this lab showed that transplanted nerves containing growing Schwann cells but lacking axons were able to induce terminal withdrawal. In these cases, AChR sites are left uncovered (Trachtenberg and Thompson, 1997). This is consistent with our observations (Fig. 2.17). Furthermore, nerve terminal loss is progressive and preceded changes in AChR density following applications of GGF on muscles development, which are believed to produce through Schwann cells (Trachtenberg and Thompson, 1997). Therefore, we suggest that denervated Schwann cells and their processes may cause regenerating nerve terminals to withdraw from endplates and further induce AChR loss.

We did observe loss of polyneuronal innervation causing AChR loss, but these observations were few. There are several possible reasons. First, we were not able obtain many cases of polyneuronal innervation following single crush on muscles. Our in vitro results show that 9 days following single crush, less than 25% of the endplates are polyneuronally innervated, in agreement with previous observation that about 20% endplates were polyneuronally innervated (Rich and Lichtman, 1989). However, in our in vivo results, there may be even less

polyneuronal innervation since we focused on the endplates with Schwann cell processes or escaped fibers. Second, we might miss some transient polyneuronal innervations in our experiments since we paid more attention to neuromuscular junctions prior to nerve regeneration and at the beginning of nerve regeneration. Meanwhile, in some cases we might not be able to identify one or two axons in one old Schwann cell tube.

Finally, resection of the nerve in reinnervated muscles can block AChR loss following nerve regeneration, suggesting loss of AChR is due to interactions between inactive receptors (denervated) and active receptors (innervated) (Fig 2.17). This is in agreement with previous work that blocking a portion AChRs with high concentration bungarotoxin cause AChR loss at these sites (Balice-Gordon and Lichtman, 1994). (See more in Conclusion)

CHAPTER 3

Schwann cells Induce and Guide Nerve Sprouting in Partially Denervated Soleus Muscles *in vivo*

ABSTRACT

The neuromuscular junctions (NMJs) in soleus muscles of transgenic mice that express GFP under control of the S100 promoter and CFP under control of the Thy-1 promoter have been viewed vitally in a repetitive fashion using a fluorescence microscope and techniques established by Lichtman and his colleagues. The cell bodies and processes of Schwann cells as well as nerve fibers and nerve terminals in these mice can be visualized by their GFP and CFP fluorescence respectively. Following partial denervation, I found that terminal Schwann cells commonly extended long processes at denervated endplates. Moreover, some of these Schwann cell processes formed Schwann cell bridges interconnecting innervated and denervated endplates, which preceded nerve terminal sprouting following partial denervation. Therefore, nerve terminal sprouts grow to innervate adjacent, denervated endplates by following the Schwann cell processes. I also found that loss of acetylcholine receptor sites occurred frequently at endplates that extended terminal sprouts but not at innervated endplates in which terminal sprouts were unsuccessful in innervating denervated endplates long-term following partial denervation. Further, I observed cases of nodal sprouting where sprouts crossed from nodes of endoneurial tubes with axons to adjacent tubes without axons and then innervated denervated

endplates in some cases. The crossing sites are always where these innervated and denervated endoneurial tubes align together and associated with Schwann cell processes. Taken together, these results suggest Schwann cell processes have the ability to induce and guide nerve sprouting.

METHODS

Animals

Double transgenic mice were used in most of our experiments. These mice most commonly expressed two, soluble, cytoplasmic fluorescent protein- (FP-) reporters: EGFP (enhanced green fluorescent protein) driven by the S100 promoter in Schwann cells (manuscript in preparation), and CFP (cyan fluorescent protein) driven by the thy-1 promoter in motor neurons (Feng et al., 2000). In a few experiments the mice employed were doubly transgenic for thy-1-CFP and GFP driven by nestin promoter and neural enhancer (Mignone et al., 2003 and manuscript in preparation). These GFP-nestin mice mirror the expression of nestin itself in Schwann cells: nestin is undetectable by immunostaining in Schwann cells in intact nerves and at innervated junctions, but is rapidly up-regulated following denervation (ref). All mice were derived by inbreeding of C57 BL6 and DBA strains and no effort was made to breed to minimize differences in genetic background. Genotyping of FP-mice was initially by Southern blotting or PCR analysis but in later experiments was accomplished by observing the mice (or a small punch from the ear) under a fluorescence dissecting microscope (manuscript in preparation). In most cases mice were bred

to homozygousity, but most animals used in the experiments were progeny that bore a single copy of each transgene insertion.

Surgery

Young adult, FP-mice (2-3 months of age, both sexes) were anesthetized by an initial intraperitoneal injection (6 µl/gm body weight, 17.3 mg/ml ketamine and 2.6 mg/ml xylazine in 0.9 % NaCl sol.) of ketamine/zylazine mixture. During imaging, a second maintenance dose of 4 µl/gm body weight was administered. The anesthesized animal was placed in a prone position on a magnetic, stainless steel plate and secured with rubber bands attached to its limbs and to magnets that could be positioned on the plate. The right hindlimb was positioned with its lateral surface uppermost, hair was removed with a depilatory, and a midline incision through the skin was made in the shank. To expose the soleus muscle, it was necessary to make a midline incision into the gastronemius muscle, cutting a few muscle fibers where it inserts on the fascia of the anterior compartment. The soleus muscle and its nerve were exposed by retracting the wound opening and retracting the lateral gastrocnemius posteriorly. The exposed muscle was rinsed with sterile lactated Ringers and immersed in the same fluid. A segment (about 1mm) of the nerve which is about 5 mm from the muscle is divided into two parts by a fine pin and then partial denervation is accomplished by resection on one part of the segment with iridectomy scissors.

In Vivo Imaging

Imaging of soleus was conducted using modifications of these techniques and those of Wigston (Wigston, 1989). The soleus was exposed as described above for the denervation and the plate to which the animal was attached was secured to the stage with an upright epi-fluorescence microscope (Zeiss Axiotech). Exposure and manipulation of the margins of the incision and the muscle were accomplished with retractors and manipulators attached magnetically to the base plate. A metal rod with its end flattened to the shape of a small spatula was used to lift the soleus muscle by pressing this spatula to the external surface of the medial side of the shank. A volume of lactated Ringers containing 2 μg/ml of rhodamine-conjugated alpha-bungarotoxin sufficient to cover the muscle was applied for 4-5 min. The muscle was then rinsed with lactated Ringers. A small, oval piece of coverglass was cut from a #0 piece of glass to 2-3 mm in its longest axis and its edges firepolished. This oval coverglass was glued to the end of a small rod held in a magnet-anchored micromanipulator, oriented orthogonal to the optical axis of the microscope, and lowered into position so it touched the surface of soleus and depressed and flattened the lateral edge of the muscle. After images were collected, the gastrocnemius muscle was moved back into position and the wound was sutured using 6-0 silk suture. The animal was allowed to recover under a heat lamp and then returned to its home cage.

Immunohistochemistry and In vitro imaging

For some experiments, the muscle was removed after the final imaging session and fixed in 4% Paraformaldehyde in PBS (phoasphate buffered saline, pH 7.4) for 40 minutes. The muscle was then rinsed in 3 changes of PBS for 10 min each. A thin layer of fibers was then dissected from the surface of the muscle and mounted in fluorescence mounting medium. The same endplates imaged in vivo were located in vitro based upon the label for AChR.

I also labeled the junctions with monoclonal anti-SV2 antibody to identify the active part of the junctions. In this case, soleus muscles were fixed in 4% paraformaldehyde for 10 minutes and rinsed using PBS for 30 minutes. Muscles were then permeabilized using 100% MeOH for 10 minutes (-20^{o}C) and rinsed for 30 minutes. Muscles were then blocked in the blocking solution which was PBS containing 0.3% Triton X-100, 0.2% bovine serum albumin and 0.1% sodium azide for 30 minutes. Muscles were then incubated in the blocking solution containing primary antibodies overnight at room temperature. The following primary antibody was used: a monoclonal antibody to the synaptic vesicle protein SV-2 (Developmental studies Hybirdoma Bank, diluted 1:500) to visualize the nerve terminal. AChRs were visualized using Cy5-conjugated α-bungarotoxin. After the incubation in primary antibodies, muscles were then rinsed in the blocking solution for 30 minutes. Muscles were then incubated in the blocking solution containing secondary antibodies for 1 hour. The secondary antibody for SV-2 and 2H3 was fluorescein-conjugated sheep $F(ab')_2$ fragment anti-mouse

(Sigma, F-2266), diluted 1:100. After the incubation in secondary antibodies, muscles were rinsed in PBS for 30 minutes.

Microscopy and Image Capture

The NMJs were imaged using a Zeiss Axiotech epi-fluorecence microscope with water immersion objectives (10X 0.3NA; 40X 0.75NA; 63X 0.9NA) or, a Leica MDRX epi-fluorescence microscope with Nikon water immersion objectives (10X 0.3NA; 40X 0.8 NA). Illumination from a 100 watt mercury source was attenuated by neutral density filters (most commonly transmittance 1-10% for TRITC, 1-25% for GFP, and 1-50% for CFP). Various filters and dichroics (Chroma) were inserted for illuminating and viewing the different fluorochromes: TRITC 535/50x, 565LP, 610/75m; GFP 495/30x, 515LP, 535/30m; CFP 436/20x, 455DCLP, 470/40m. The filters and dichroics for GFP and CFP were designed to reduce the cross-over in fluorescence excitation and emission for these two FPs at some cost of signal intensity; they worked very well except for cases where the GFP fluorescence was much brighter than that for CFP, which happened rarely in our experiments.

The images were acquired using a sensitive CCD camera, a Princeton MicroMax 5MHz (Roper Sci.). A Macintosh computer with IP Lab software (Scanalytics, Fairfax, VA) was used to control the camera and an electronic shutter in the illumination path. Images were commonly collected with 1 sec exposures with the cameras set to bin at 2X2 (image of 650 X 515 pixels).

RESULTS

In response to partial denervation, terminal Schwann cells extend Schwann cell processes from endplates

Son and Thompson（1995）have reported terminal Schwann cells grew Schwann cell processes at both denervated and innervated endplates following partial denervation. To investigate terminal Schwann cell process growth in our double transgenic mice, I partially denervated soleus muscles in 5 mice and examined the muscles in vitro 4 days later. Terminal Schwann cell processes were found at both denervated and innervated endplates, but it appeared that the processes at innervated endplates were different from the numerous and long Schwann cell processes at denervated endplates. Of 196 denervated endplates examined, 180 endplates had Schwann cell processes (of average length 21.3±1.0μm (n=104)). However, 24 of 85 innervated endplates extended shorter Schwann cell processes (of average length 7.6±0.7μm (n=25)) significantly different from denervated endplates (p<0.001). Further, individual denervated endplates extended significantly more Schwann cell processes (on average 2.3±0.1 per endplate (n=88)) than innervated endplates (on average 1.2±0.1 per endplate (n=24)) (p<0.001). Therefore, we conclude that both denervated and innervated endplates extend Schwann cell processes following partial denervation; but Schwann cell processes at denervated endplates are more robust.

Schwann cell processes from denervated endplates form Schwann cell bridges interconnecting innervated and denervated endplates

In some cases, we found the Schwann cell processes grew to interconnect two endplates following partial denervation. These processes are named Schwann cell bridges. These bridges were further found between denervated and innervated endplates in most cases, consistent with previous observations (Love and Thompson, 1999). In 3 soleus muscles 4 days after partial denervation, I found that 30 of 35 Schwann cell bridges were between denervated and innervated endplates, only 5 bridges were found between denervated endplates. To investigate how Schwann cell bridges form, I extended my observations to the behavior of the Schwann cell processes in a small area of the muscles, in which there were denervated endplates and innervated endplates. I first imaged NMJs in soleus muscles at early times (2 days) and then later times following partial denervation. At 18 cases in 15 soleus muscles, I was able to observe the whole processes of Schwann cell bridge formation. In all but one case, I found that Schwann cell processes from denervated endplates extended towards and then made contact with innervated endplates. An example is shown in Fig. 3.1. Moreover, in 3 of these cases, I found small Schwann cell processes from innervated endplates aligned along Schwann cell bridges which were formed by processes from denervated endplates. Only one case showed a Schwann cell process from an innervated endplate that appeared to form a Schwann cell bridge.

Further evidence that Schwann cell processes from denervated endplates formed Schwann cell bridges were obtained from Nestin-GFP transgenic mice

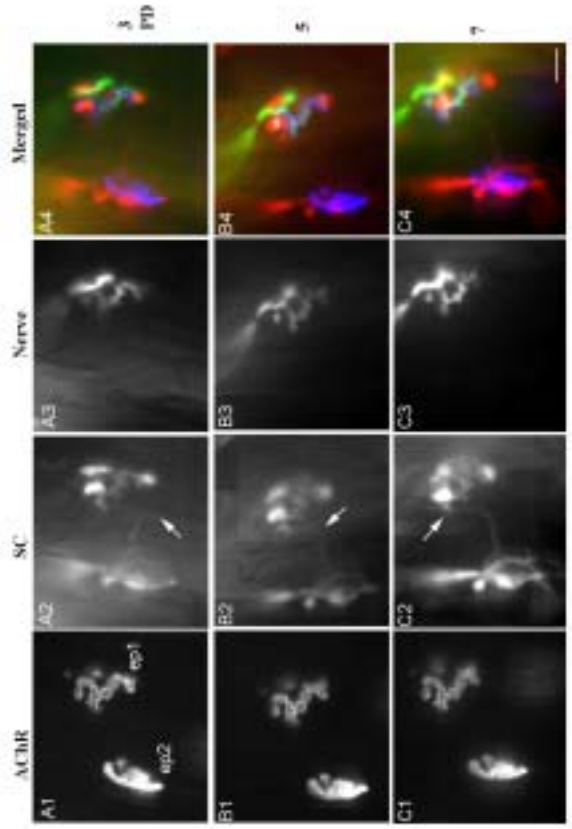

Figure 3.1: Schwann cell processes from denervated endplates form Schwann cell bridges that connect to nearby innervated endplates

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves were viewed repeatedly at 2 endplates (ep1 and ep2) over the course of 65 days following partial denervation. In the merged images (A4, B4 and C4), AChRs, SCs and nerves appear as blue, red and green, respectively. When the first images were taken on day 3 following partial denervation, ep2 was denervated but ep1 remained innervated. At this time, Schwann cells at ep2 had extended a process towards the middle of the panel (arrow in A2). This SC process had changed direction and reached the Schwann cells of ep1 by day 5 (arrows in B2 and C2). This Schwann cell "bridges" was occupied by a terminal sprout from ep1 when the next view was made on day 25 (not shown). Moreover, the bridge and sprout persisted at the last observation on day 65 (not shown).

that expressed GFP in Schwann cells and their processes after the degeneration of axons. I was able to obtain the whole processes of Schwann cell bridge formation in 6 cases in 6 Nestin-GFP transgenic mice. The Schwann cell processes from denervated endplates labeled by GFP formed all but 1 Schwann cell bridge (Fig 3.2). This one case show that Schwann cell processes from the denervated endplate covered about 70% of the Schwann cell bridge which was labeled by S100 antibody later on in vitro (Fig 3.3).

We conclude therefore, that both denervated and innervated endplates extend Schwann cell processes in response to partial denervation; however Schwann cell processes from denervated endplates form Schwann cell bridges in most cases and processes from innervated endplates may also be involved in Schwann cell bridge formation in a few cases.

I do not have enough evidence to determine whether there is a specific orientation of Schwann cell growth. However we don't believe that Schwann cell processes grow randomly at all denervated endplates. First, the real distance between those endplates I observed may be different from what I view under the microscope. For example, two endplates could be located at different sides of two muscle fibers so that the real distance was much longer than that apparent in the microscope. Moreover, some cases show Schwann cells extended processes across muscles fibers and connect to adjacent innervated endplates, ignoring other Schwann cell processes growing along muscle fibers at these endplates (Fig. 3.1). Therefore, we suggest that initial Schwann cell process growth at denervated endplates is random, but there is orientation of this process growth when these

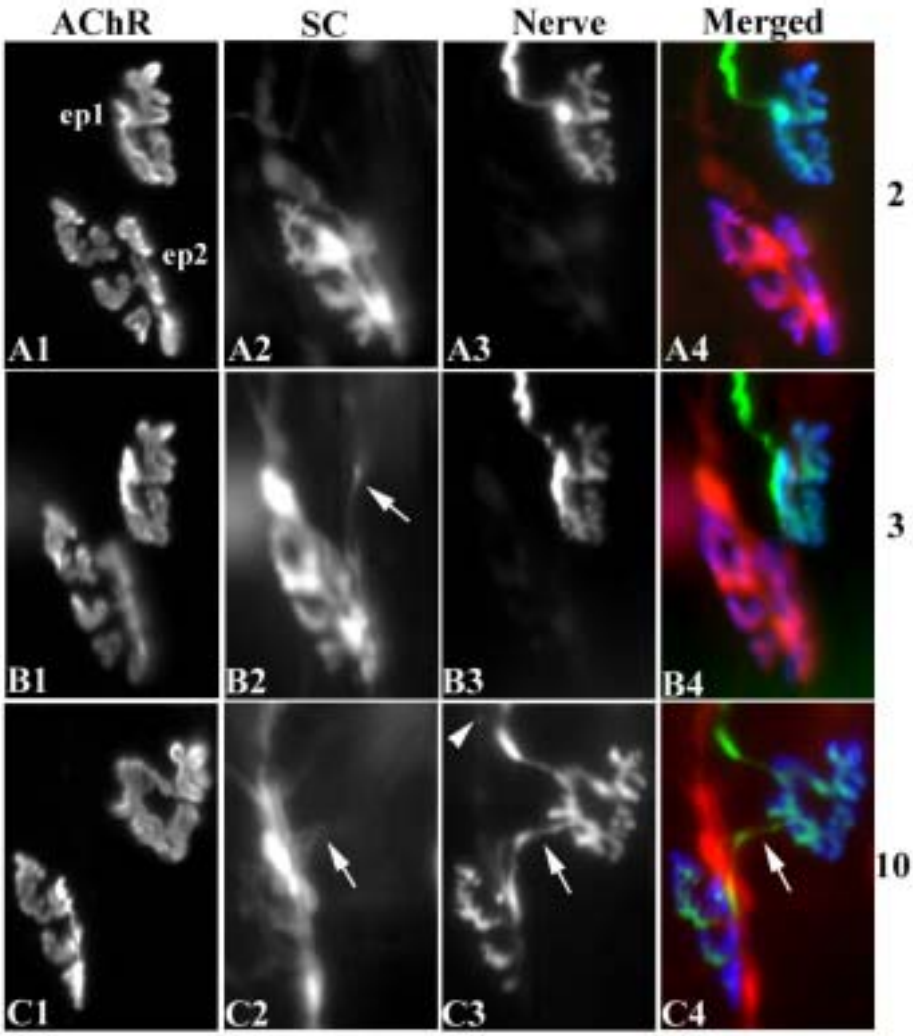

Figure 3.2: Schwann cell processes from denervated endplates form Schwann cell bridges which induce and guide terminal sprouts

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves were viewed repeatedly at 2 endplates (ep1 and ep2) over the course of 10 days following partial denervation. In the merged images (A4, B4 and C4), AChRs, SCs and nerves appear as blue, red and green, respectively. When the first images were

collected on day 2, the expression of the nestin-promoter-driven fluorescence in the SCs of ep2 (A2) and the presence of a nerve terminal over ep1 (A3) show that ep1 is innervated and ep2 denervated. The GFP-expressing SCs at ep2 have extended a process towards and reached ep1 (arrows in A2 and A4) by day 3. Ep2 had extended a terminal sprouts along the SC process to innervate ep1 (arrows in C2, C3 and C3) and this terminal sprout continued to grow retrogradely over the old SC tube of ep1 (arrowhead in C3) by day 10. Following dissection and fixation of the muscle, in vitro observations were performed and same results were obtained (not shown).

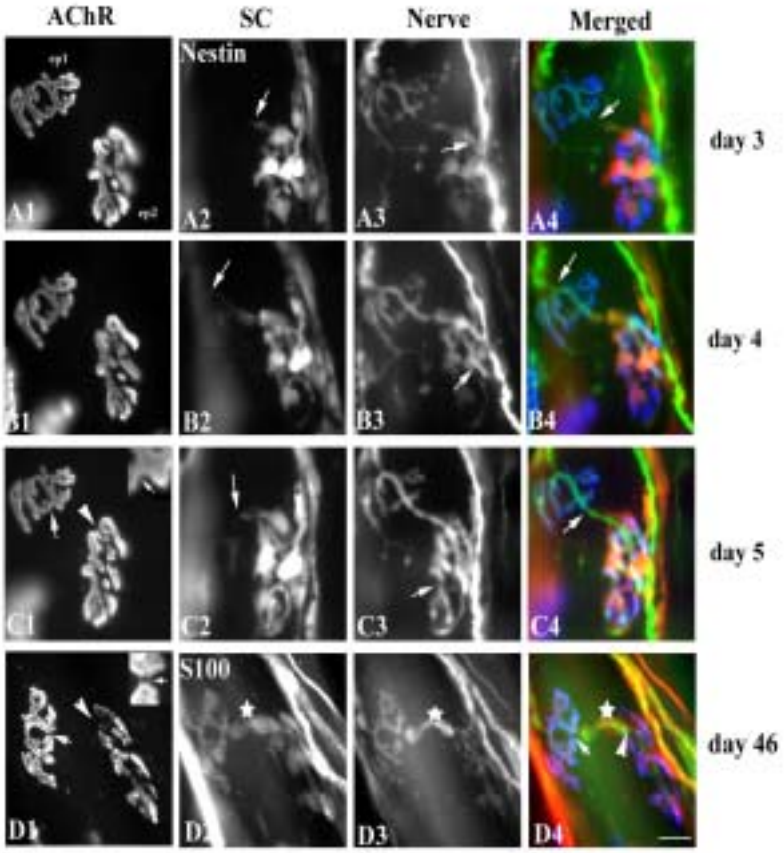

Figure 3.3: In some cases, Schwann cells growing from both innervated and denervated endplates participate in the formation of Schwann cell bridges following partial denervation

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at 2 endplates (ep1 and ep2) were viewed repeatedly over the course of 46 days following partial denervation. In the merged images (A4, B4, C4 and D4), AChRs, SCs and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day 2 and 46. When the first images were collected on day 3, the expression of the nestin-promoter-driven fluorescence in the SCs of ep2 (A2) and the presence of a nerve terminal over ep1 (A3) show that ep1 is innervated and ep2 denervated. The GFP-expressing SCs at ep2 have extended a process towards ep1 (arrow in A2) but this process has covered only a portion of the distance between the endplates. A terminal sprout from ep1 has already grown by day 3 to

contact a portion of ep2 (arrow in A3). The SC process from ep2 (arrow in A2) is projecting along the course of this terminal sprout. Other observations have shown that the entire length of such sprouts is covered by a SC bridge. Therefore, the remainder of the distance between the endplates must be covered by SC processes extending from ep1. Thus, these observations suggest that SC processes from both ep2 and ep1 have formed the bridge on day 3. During the time between day 4 and day 5, the SC process from ep2 reaches and then withdraws from ep1 (compare arrow in B2 and C2). This occurs as the terminal sprout occupies more regions of ep2 and becomes thicker (compare arrow in B3 and C3). The last images on day 46 were obtained in vitro after fixation of the muscle and application of an antibody to the protein S100 to visualize SCs. There is a clear SC bridge between these two endplates that is associated with the terminal sprout (asterisk in D2, D3 and D4). Moreover, it appears that some AChR sites have been lost from ep1 (arrow in D1, and compare arrow in insets showing larger views of the sites where AChR have been lost). Also it appears that some receptor sites have been lost from ep2 (arrowheads in D1). These sites of receptor loss are sites where the terminal sprout leaves ep1 and enters into ep2 (arrow and arrowhead in D4). Note that by day 46 most of the sprouts extended from ep1 other than the one on the bridge have withdrawn (compare A3, B3, C3 and D3). Bar 20 μm

randomly growing processes are close to adjacent innervated endplates.

Schwann cell bridge formation precedes nerve terminal sprouting following partial denervation

In agreement with previous observations (Son and Thompson, 1995b), I found that denervated endplates were reinnervated by terminal sprouts from adjacent innervated endplates, which were always associated with Schwann cell bridges, following partial denervation. First, in our in vitro experiments, I found that 26 of 169 denervated endplates were innervated by terminal sprouts in 4 soleus muscles 4 days following partial denervation. To examine the possibility that Schwann cell bridges guide terminal sprouts to innervate adjacent endplates following partial denervation, like their behavior in nerve regeneration, soleus muscles were initially imaged 2 days following partial denervation to identify the denervated and innervated endplates and then those endplates would be repeatedly viewed at later times. In 17 soleus muscles, I observed the whole processes of the formation of 18 terminal sprouts. In all cases, these terminal sprouts grew over preexisting Schwann cell bridges to innervate adjacent endplates (Fig. 3.4). In 8 of these cases, I found that Schwann cell processes from denervated endplates formed bridges and induced as well as guided terminal sprouts to innervate these endplates (Fig. 3.5). Moreover, I also confirmed these results by using Nestin-GFP transgenic mice. In 5 Nestin-GFP transgenic mice, I was able to observe formation of terminal sprouts in 5 cases. All cases show that terminal sprouts grew over the pre-existing Schwann cell bridges formed by Schwann

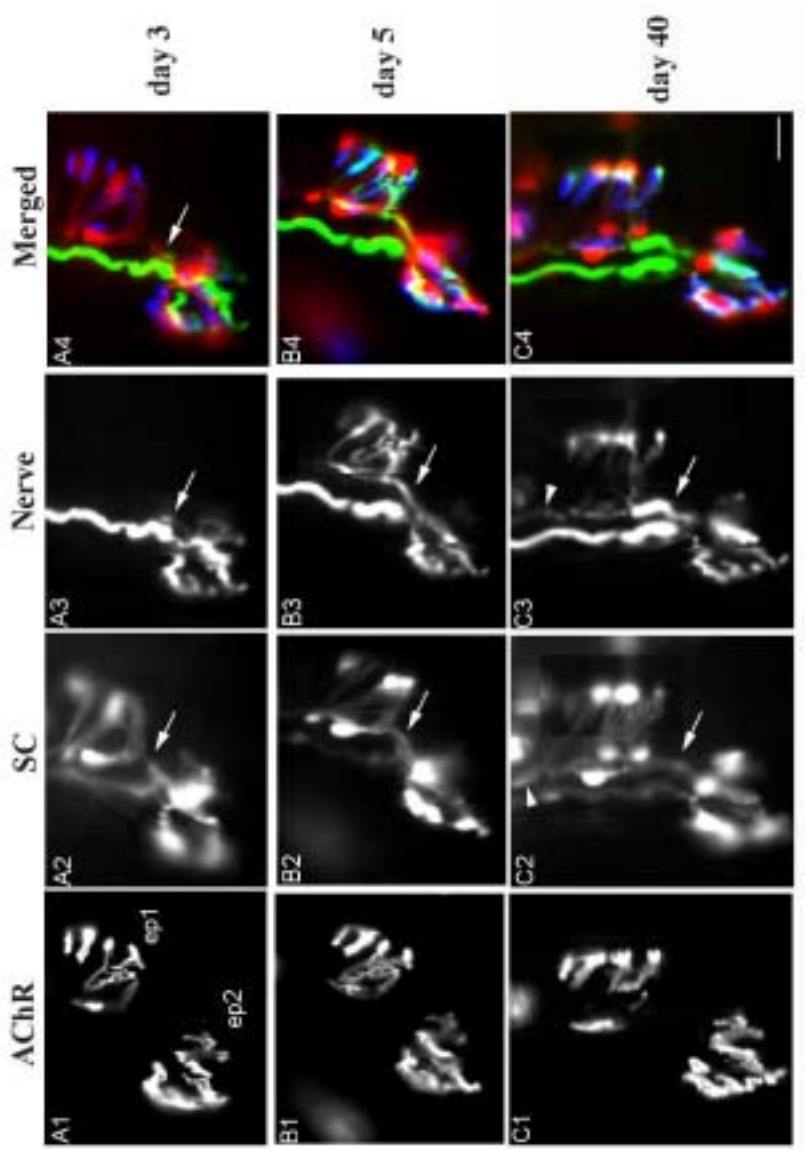

Figure 3.4: Schwann cell bridge formation precedes nerve terminal sprouting following partial denervation

Acetylcholine receptors (AChR), Schwann cells (SCs), and nerves at two endplates (ep1 and ep2 in A1) were imaged repeatedly over the course of 40 days following partial denervation. In the merged images (A4, B4 and C4), AChR, SCs

and nerves appear as blue, red, and green, respectively. At the time the first images were collected on day 2, ep2 is innervated but ep1 is denervated (compare A1 and A3). There is a SC bridge interconnecting the two endplates (arrow in A2), a small sprout from the axon innervating ep2 appears to be growing on this bridges (arrow in A3) but does not contact ep1. By day 4, this terminal sprout has grown from ep2 to innervate ep2 (arrow in B3). The SC bridge and terminal sprout persisted for at least 35 days (arrows in C2 and C3). This sprout continued to grow (arrowhead in C3) up the old SC tube (arrowhead in C2) that previously contained the axon innervating ep1. Following dissection and fixation of the muscle, in vitro observations were performed and same results were obtained (data not shown). Bar 20 μm

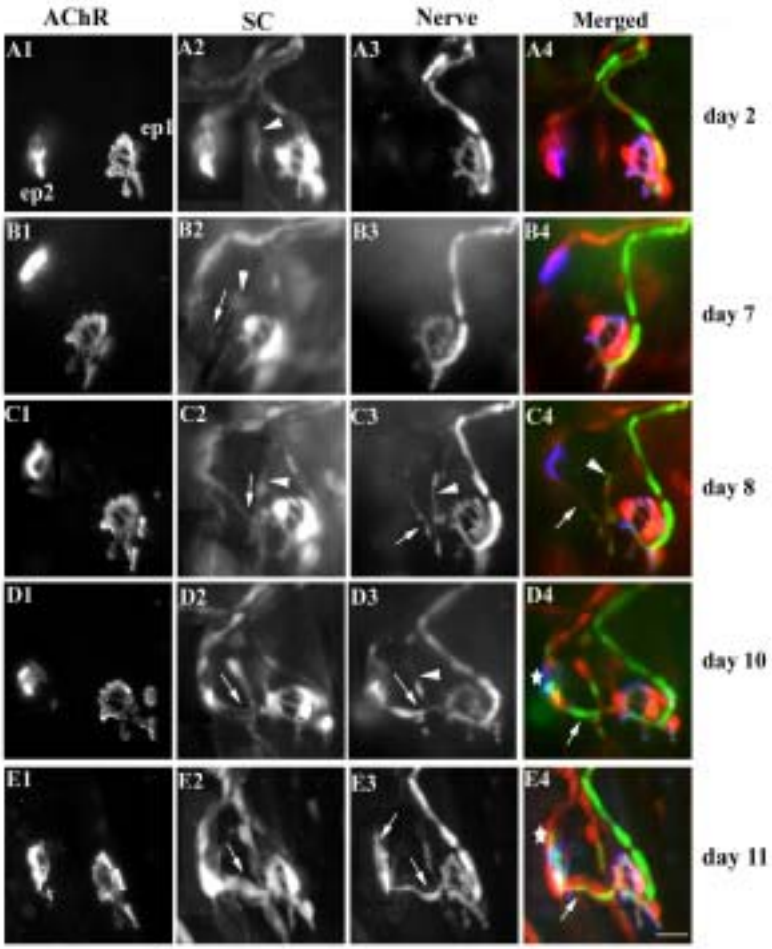

Figure 3.5: Following partial denervation, nerve terminal sprouts grow to innervate adjacent, denervated endplates by following Schwann cell processes that grow from denervated endplates

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at 2 endplates (ep1 and ep2) were viewed repeatedly over the course of 11 days following partial denervation. In merged images (A4, B4, C4, D4 and E4), AChRs, SCs and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day 2 and 10. When the first images were collected on day 2

following partial denervation, ep1 remains innervated, but ep2 is denervated (compare A1 and A3). At this time, there is no SC process connecting these two endplates (A2). Five days later, on day 7, ep2 has extended a SC process towards ep1 (arrow in B2), but no terminal sprout is yet present. By day 8, a Schwann cell process had connected the two endplates and formed a Schwann cell bridge (arrow in C2 and C4). A terminal sprout has extended from ep1 over the SC bridge (arrow in C3 and C4). Moreover, the terminal sprout also extended towards the top of the image along a SC process (arrowhead in C3 and C4) which was present even on day 2 and met the SC bridge on or before day 7 (compare arrowhead in A2, B2 and C2). By day 10, the terminal sprout has grown to innervate ep2 (arrow in D3 and asterisk in D4). In addition, the branch of the sprout directed upwards in the image in C3 has partially withdrawn (compare arrowhead in C3 and D3). In the last images collected in vitro after sacrifice of the animal on day 11, the terminal sprout innervating ep2 (asterisk in E4, arrows in E3) and the associated SC bridge (arrow in E2) are clear. Bar 20 μm

cell processes from denervated endplates, labeled by GFP. An example is shown in Fig 3.2. In a number of cases, I also found that one axon could innervate several endplates through Schwann cell bridges (Fig. 3.6).

However, I also found that innervated endplates did not extend terminal sprouts over preexisting Schwann cell bridges interconnecting innervated and denervated endplates in some cases. In 7 soleus muscles, I found no or no stable terminal sprouts extending from innervated endplates over 8 Schwann cell bridges. Interestingly, in 7 of these cases, the denervated endplates were reinnervated by other axons that arrived over old Schwann cell tubes around the time when these Schwann cell bridges were formed. An example is shown in Fig. 3.7. These results suggest reinnervation at denervated endplates causes Schwann cell bridges to fail to induce terminal sprouts.

Meanwhile, I also found that innervated endplates extended Schwann cell processes; and in some cases terminal sprouts followed these processes extending out of endplates. First, all the terminal sprouts I observed were associated with Schwann cell processes. Second, in the cases I observed Schwann cell processes always preceded terminal sprouts. However, these terminal sprouts that did not connect to other endplates disappeared within about 10 days after partial denervation in most cases (Panel B and C in Fig. 3.8).

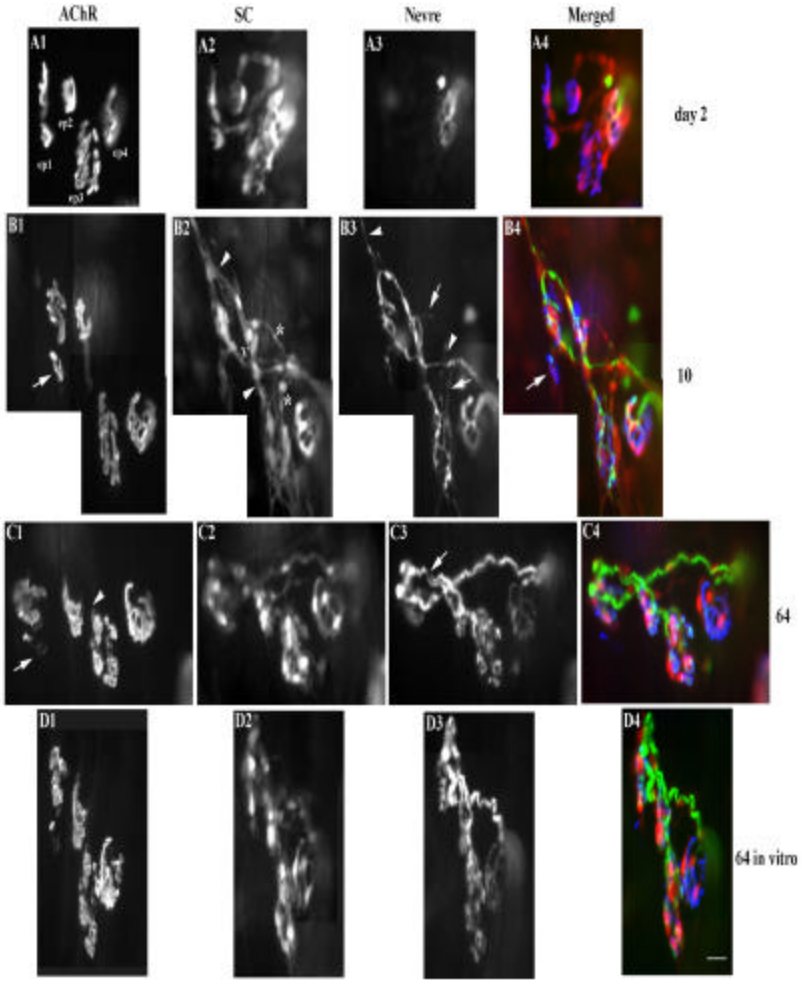

Figure 3.6: One nerve sprout innervates three endplates following partial denervation

Acetylcholine receptors (AChRs), Schwann cells and nerves at 4 endplates (ep1, ep2, ep3 and ep4) were viewed repeatedly over the course of 64 days following partial denervation. In the merged images (A4, B4, C4 and D4), AChRs, SCs and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day 2 and 64. When the first images were collected

on day 2, only ep4 remained innervated (A3). By day 10, one nerve sprout (presumably a nodal sprout) had arrived at ep1 over its old SC tube ("V" in B2) and grown to innervate ep2 and ep3 over SC bridges (asterisks in B2). Also at day 10, there are at least two escaped fibers associated with SC processes (arrowheads in B3) and two associated with old SC tubes of ep2 and ep3 (arrows in B3). Note some AChR sites in ep1 are poorly occupied by the nerve (arrow in B1 and B4). On day 64, the innervation pattern at these endplates persists, except for the appearance of a new nerve branch from the nerve sprout connecting ep2 and ep3 (arrow in C3). All escaped fibers had withdrawn, including those previously associated with old SC tubes. SCs that were previously associated with nerve growths (except for those in the SC bridges) had also disappeared. Faintly labeled AChR are found at the site in ep1 which had been only partially covered by the nerve on day 10 (compare arrow in B1 and C1). There is also AChR loss from the site of entry of the nerve sprout into ep3 (arrowhead in C1). Note no AChR loss at ep4 which remains innervated. Images were also obtained in vitro (D panels) following removal and fixation of the muscle; the same observations as in panel C were made. Bar 20 μm

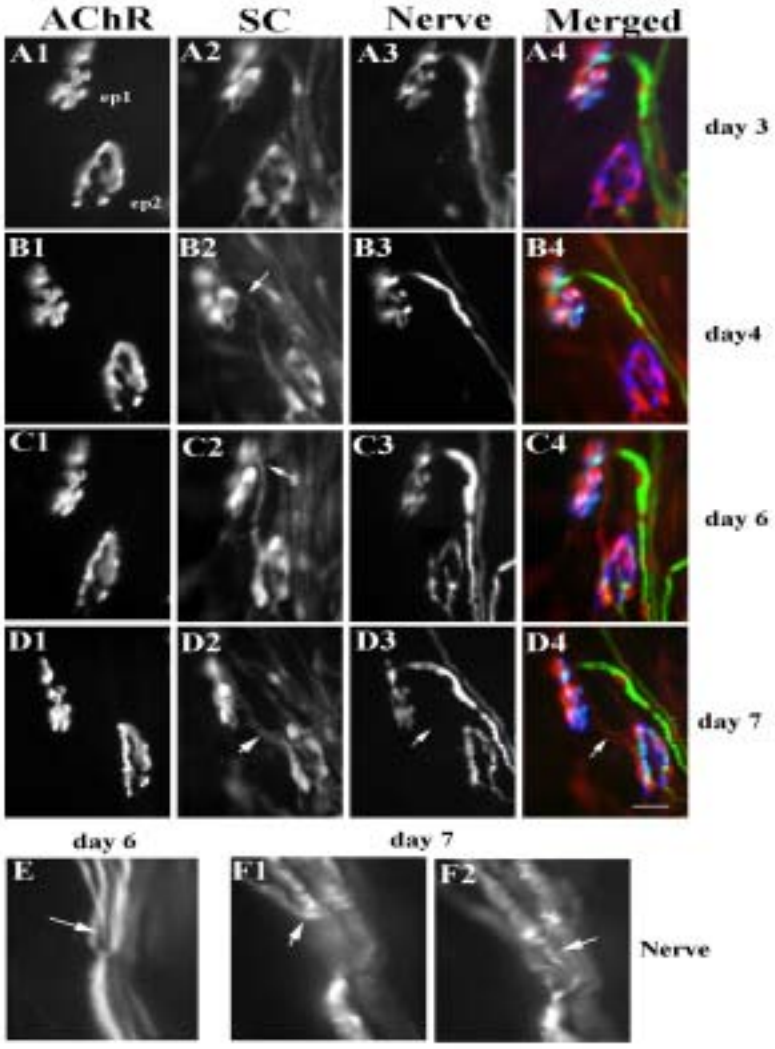

Figure 3.7: Schwann cell bridges do not always lead to sprout formation

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at 2 endplates (ep1 and ep2) were viewed repeatedly over the course of 7 days following partial denervation. In the merged images (A4, B4, C4 and D4), AChRs, SCs and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day 3 and 7. When the first images were collected on day 3 (panel A), ep1 remains innervated, but ep2 is denervated. At this time, no SC process connects these two endplates. However, one day later, on day 4 (Panel B), a SC

process has grown from ep2 towards ep1 (arrow in B2). By day 6 (Panel C), this SC process has made contact with ep1, forming a SC bridge. No sprout from ep1 is evident. A sprout (arrow in D3 and D4) remains absent on day 7, despite the persistence of the bridge (arrow in D2). Note however that ep2 became reinnervated by an axon from the bottom of the panel (C3) sometime between day 4 and day 6 (C3). Examination of microscopic fields below panel C3 shows that this axon comes from a nodal sprout from within the intramuscular nerve (arrow in E). Further information about this sprout was obtained following the vital viewing on day 7, when the animal was sacrificed, the muscle was fixed and viewed in vitro. By examination at higher magnification with a high NA objective and by focusing through the site of origin (panels F1 and F2), it is clear that the sprout originates from the same axon innervating ep1 at a point where this axon bifurcates to give rise to two axonal branches (arrow in F1 and F2). Bar 20 μm

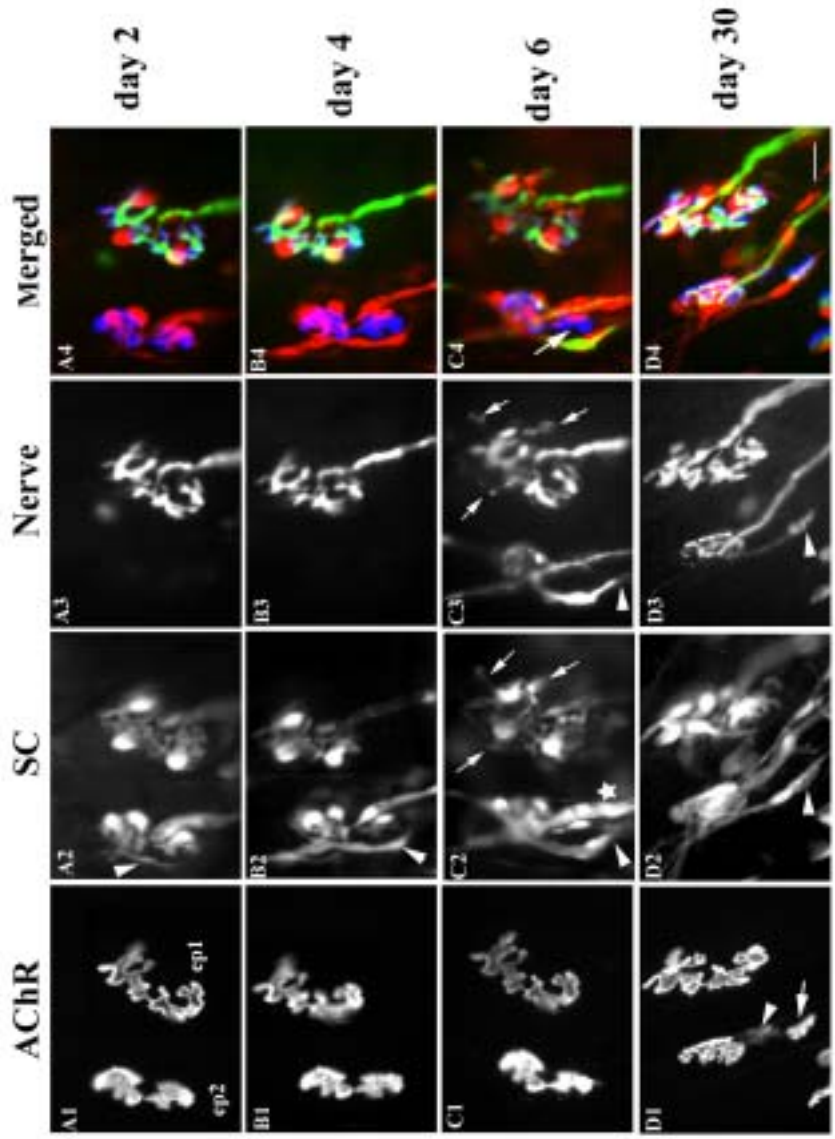

Figure 3.8: **AChR sites are lost from and added to endplates during their reinnervation following partial denervation, but innervated endplates that do not sprout to innervate adjacent endplates appear stable**

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at two endplates (ep1 and ep2) were viewed repeatedly over the course of 30 days after

partial denervation. In the merged images (A4, B4, C4 and D4), AChRs, SCs and nerve appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day 2 and 30. When the first images were collected 2 days after partial denervation, ep1 was innervated, but ep2 was denervated (compare A1 and A3). By day 2, at least one SC process had extended from ep2 (arrowhead in A2). This process appears longer and thicker by day 4 (arrowhead in B2). Two days later on day 6, ep1 had been reinnervated by a regenerating axon or a sprout arriving over the old SC tube (asterisk in C2). At this time, this axon at ep2 had generated a minimum of three escaped fibers (C3) that are associated with SC processes (C2). Note some of the AChR sites in ep2 are not occupied by the nerve at day 6 (arrow in C4). In the meantime, the SCs at ep1 at day 6 had extended at least 3 small SC processes (arrows in C2) with 3 corresponding terminal sprouts (arrows in C3). The last images were made in vitro on day 30 after the animal was sacrificed and the muscle was fixed. The nerve sprouts and some SC processes at ep1 have withdrawn (D2 and D3). No AChR sites appear to have been lost from this endplate (compare ep1 in C1 and D1). Ep2 has lost some AChR sites (arrowhead in D1) which had not been re-occupied by the nerve on day 6 (arrow in C4). Ep2 also has gained some AChR sites (arrow in D1). A Schwann cell process (arrowhead in C2 and D2) and an escaped fiber (arrowhead in C3 and D3) had been present at these sites from day 6 to 30. Thus, the Schwann cell process and the escaped fiber appear to precede the addition of new AChR sites. Bar 20 μm

Terminal sprouts are persistent and some of them grow retrogradely over old Schwann cell tubes following partial denervation

I extended our observations to investigate events after endplates were reinnervated by terminal sprouts. First, some of terminal sprouts between denervated and innervated endplates were observed long-term (42-64 days) after partial denervation to examine the stability of Schwann cell bridges and terminal sprouts. Of 12 terminal sprouts in 8 soleus muscles, 11 terminal sprouts persisted over the course of long-term partial denervation. An example is shown in Fig. 3.3. Furthermore, we found in some cases that endplates appeared to be innervated by terminal sprouts and by axons located within old Schwann cell tubes. However another possible explanation in these cases is that terminal sprouts grew in a retrogradely direction over the old Schwann cell tubes. Therefore, to understand this question, I observed the endplates during the time when denervated endplates were reinnervated by terminal sprouts and then followed these over subsequent days. I obtained images for 12 cases in 11 soleus muscles. All cases showed that terminal sprouts grew over the old Schwann cell tubes retrogradely. I was not able to trace all these terminal sprouts growing over old Schwann cell tubes; however in 3 cases I found these terminal sprouts grew over the old Schwann cell tubes and innervated other endplates down the tubes (Fig. 3.9). In a few cases, I also found cases where terminal sprouts retrogradely growing over old Schwann cell tubes retracted long-term following partial denervation.

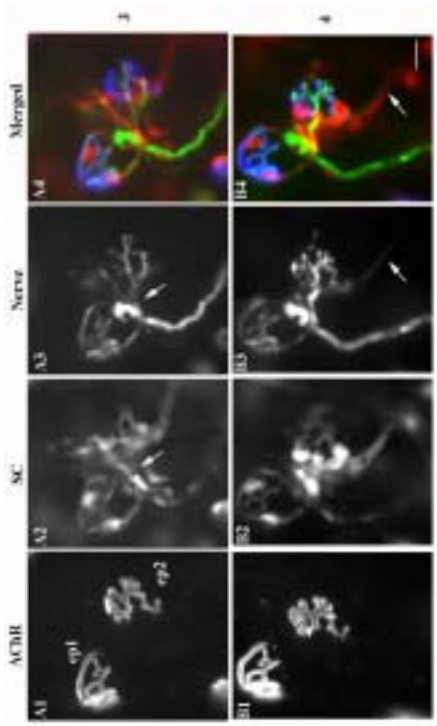

Figure 3.9: Terminal sprouts grow retrogradely over old Schwann cell tubes following partial denervation.

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at 2 endplates (ep1 and ep2) were viewed repeatedly over the course of 45 days following partial denervation. Data only for day 3 and day 4 after partial denervation are shown here. In merged images (A4 and B4), AChRs, SCs and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day3. On day 3, ep1 remained innervated (compare A1 and A3) and extended a terminal sprout over a SC bridge to innervate ep2 which was denervated (arrows in A2 and A3). One day later, the sprout grew retrogradely over the old SC tube of ep2 (arrow in C2 and C3). The terminal sprouts continued to grow over the old SC tube and innervate an endplate on another side of the old SC tube (data not shown). Bar 20 μm

We therefore, conclude that terminal sprouts persist following their growth rather than be transient. These terminal sprouts are also able to innervate other endplates by growing retrogradely over old Schwann cell tubes.

Nodal sprouts grow over old Schwann cell tubes following partial denervation.

Endplates can not be only innervated by terminal sprouts, but also by nodal sprouts growing from nodes of Ranvier in axons lacking myelinating Schwann cells (Brown et al., 1981). To examine how nodal sprouts form, I repeatedly observed nodes of the axons left following partial denervation in living transgenic mice. I found nodal sprouts grew over adjacent old Schwann cell tubes leading to the denervated endplates (n=4 in 4 soleus muscles) (Fig. 3.10) or retrogradely to CNS (n=1) (Fig. 3.11). These four nodal sprouts share the following common properties. First, all nodal sprouts were found to be associated with Schwann cell processes. Second, nodal sprouts occurred in the intramuscular nerve close to denervated endplates. Third, the nodes generating sprouts were close to the old Schwann cell tubes leading to denervated endplates. In 2 cases, I further found there were small Schwann cell processes between the denervated and innervated Schwann cell tubes although these two Schwann cell tubes were close to each other. In one case I observed a small Schwann cell process growing from a denervated Schwann cell tube by using a Nestin-GFP transgenic mouse (Fig. 12). Therefore, these results suggest that Schwann cells are also crucial for generation and growth of nodal sprouts.

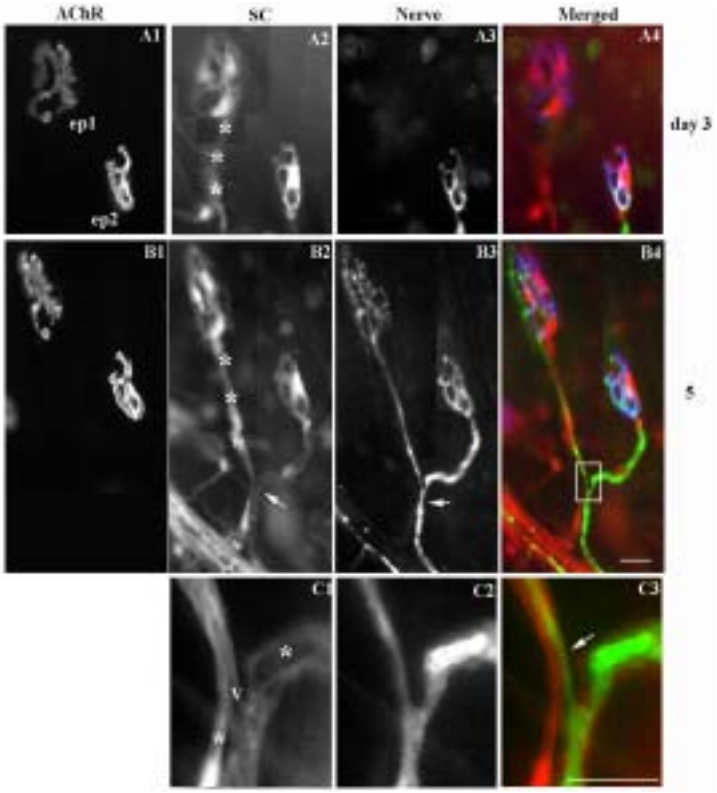

Figure 3.10: Nodal sprouts grow over old Schwann cell tubes to innervate endplates following partial denervation

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at 2 endplates (ep1 and ep2) were viewed repeatedly over the course of 45 days following partial denervation. In merged images (A4, B4 and C3), AChRs, SCs and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day3. When the first images were collected 3 days following partial denervation, ep1 was denervated but ep2 remained innervated (compare A1 and A3). The old SC tube of ep1 is shown by three asterisks in A2. 2 days later, ep1 had been reinnervated by a nodal sprout growing over its old SC tube (asterisks in B2). The sprout arose from the axon innervating ep2 (arrows in B2 and B3). Panel C shows the box in B4 at higher magnificent. There is a SC process marked as "V" in C1 interconnecting the two old SC tubes (asterisks in C1) and the process exactly aligns with the nodal sprout (arrow in C3). Bar 20 μm

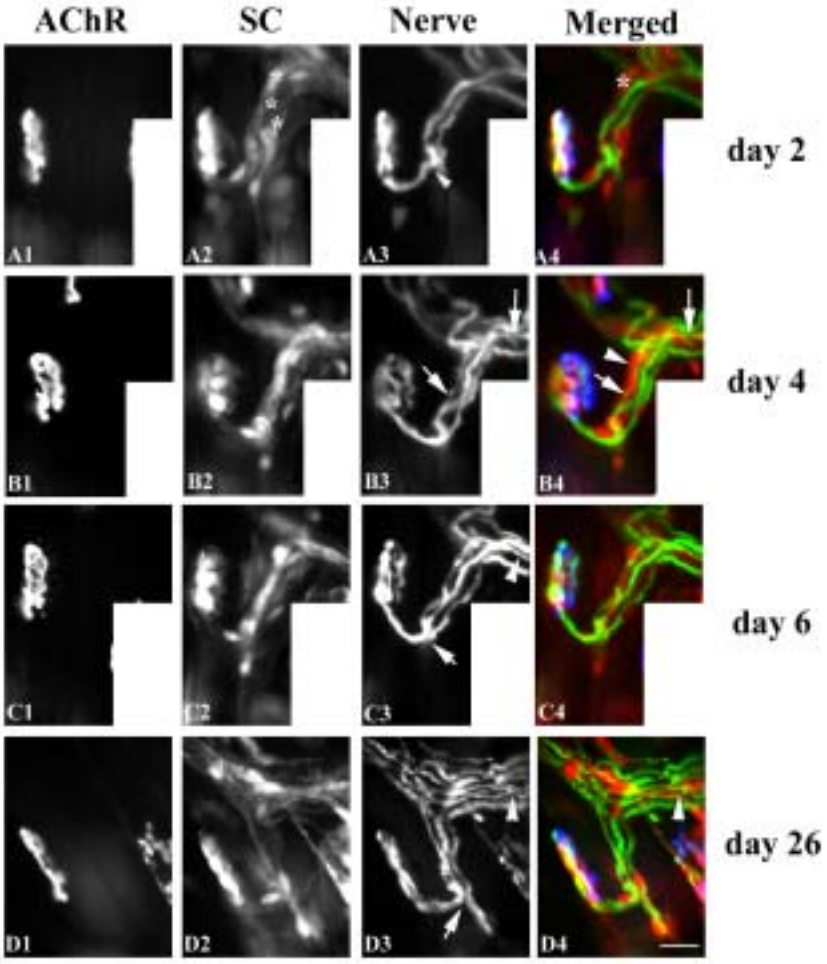

Figure 3.11: Nodal sprouts grow over old Schwann cell tubes following partial denervation

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves were viewed repeatedly in a small area in the vicinity of an endplate over the course of 26 days following partial denervation. In the merged images (A4, B4, C3 and D4), AChRs, SCs and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on day 2 and 26. When the first images were collected 2 days after partial denervation, two SC tubes (bottom two

asterisks in A2) remain filled with axons and one of these innervates the endplate in A1-D1. There is however a third SC tube present here that initially lacks an axon (upper asterisk in A2 and asterisk in A4). Two days later, on day 4, a nodal sprout (lower arrow in B3 and B4) is present that appears to have grown from the node indicated by the small arrowhead in A3. This nodal sprout has grown in the retrograde direction towards the CNS by following the vacant SC tube (arrowhead in B4). This nodal sprout has grown to the right arrow in B3 and B4 on day 4. This retrograde growth continued in the images made on day 6 (the arrowhead in C3 marks the nodal sprout as it exits the right of the panel). On day 26, the animal was sacrificed, the muscle dissected, fixed and viewed in vitro. These images show the sprout comes from a node of the axon innervating the endplate in D1. The nodal sprout remains and still projects retrogradely in the muscle nerve (arrowhead in D3 and D4). Bar 20 μm

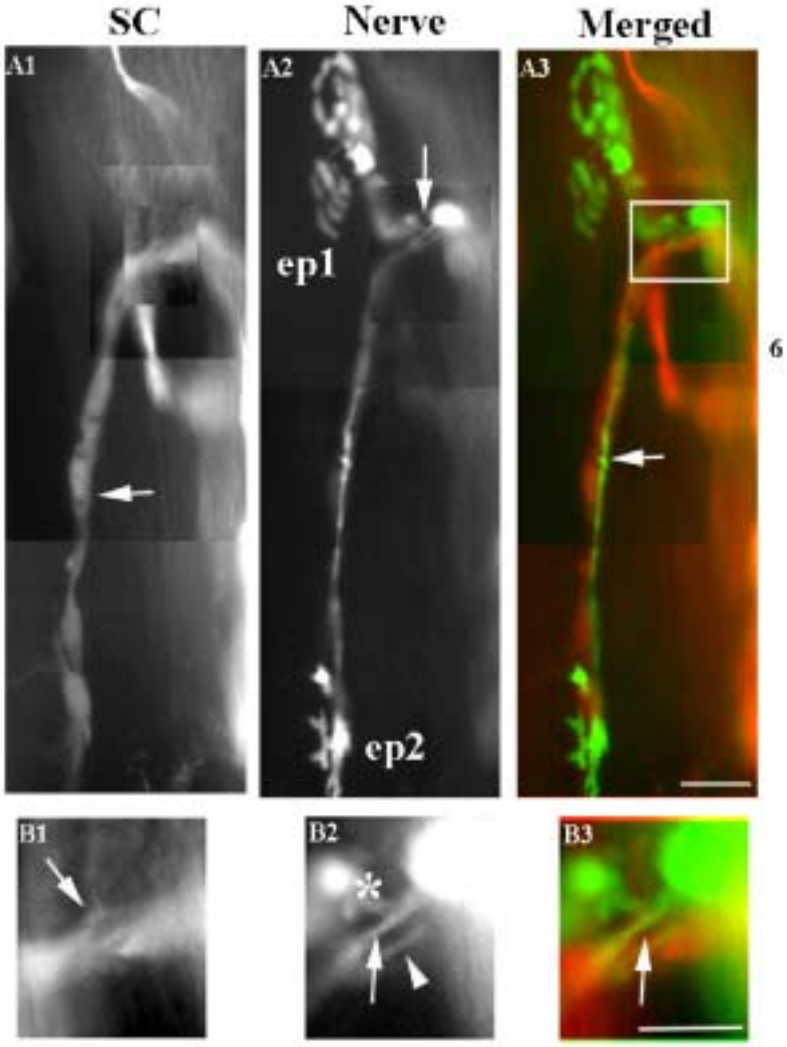

Figure 3.12: A nodal sprout jumps between two Schwann cell tubes in association with Schwann cell processes

Acetylcholine receptors (AChRs) (not shown), Schwann cells (SCs) and nerves at 2 endplates (ep1 and ep2) were viewed repeatedly over the course of 6days following partial denervation. In merged images (A3 and B3), SCs and nerves appear as red and green, respectively. New rhodamine-bungarotoxin was applied on day2 (not shown). When the first images were collected on day 2, ep1 remained innervated and ep2 was denervated (data not shown). 4 days later, a

nodal sprout extended from the axon innervating ep1 (arrow in A2) and grew and innervated over the old SC tube leading to ep2 (arrows in A1 and A3)and innervated ep2. Panel B shows the box in A3 at higher magnification. The nodal sprout is aligned with is a SC process which appears to have grow towards the node (compare arrows in B1, B2 and B3). Note: The original axon innervating ep1 marked by an asterisk in B2. An axon reinnervate one endplate out of focus shown by a arrowhead in B2 too. There is also seen in B2 yet another axon (marked by arrowhead) that reinnervates yet another endplate that is in a different focal plane. Bar 20 μm

Loss of acetylcholine receptor sites occurs at endplates that extend or receive terminal sprouts and at few innervated endplates in which terminal sprouting is transitory

I found AChR loss in partially denervated muscles and this loss did not only occur in reinnervated endplates but also in innervated endplates long-term following partial denervation. Further, I observed that AChR loss at innervated endplates was associated with extending terminal sprouts. Meanwhile, the denervated endplates receiving terminal sprouts also lost their AChRs (Fig. 3.13). In some cases, I labeled the same NMJ with anti-synaptic vesicle antibody to further understand how AChR loss occurred, after the muscles were removed after last in vivo images. Interestingly, there were no synaptic vesicles at where terminal sprouts extended out long-term following partial denervation (Fig. 3.14).

However, innervated endplates without persistent terminal sprouts did not lose AChRs. To examine the possibility that AChR loss is related to innervated endplates extending terminal sprouts long-term following partial denervation, AChRs, Schwann cells and nerves were initially imaged 2 days to identify innervated and denervated endplates, a few days later to check if there were terminal sprout formation, and then after another 40-60 days following partial denervation. In 11 soleus muscles following partial denervation, AChR loss was found at 9 of 11 innervated endplates extending terminal sprouts. These terminal sprouts were persistent. Moreover, 8 of 9 endplates receiving terminal sprouts lost their receptors in 9 muscles. In contrast, in these muscles we found 8 endplates

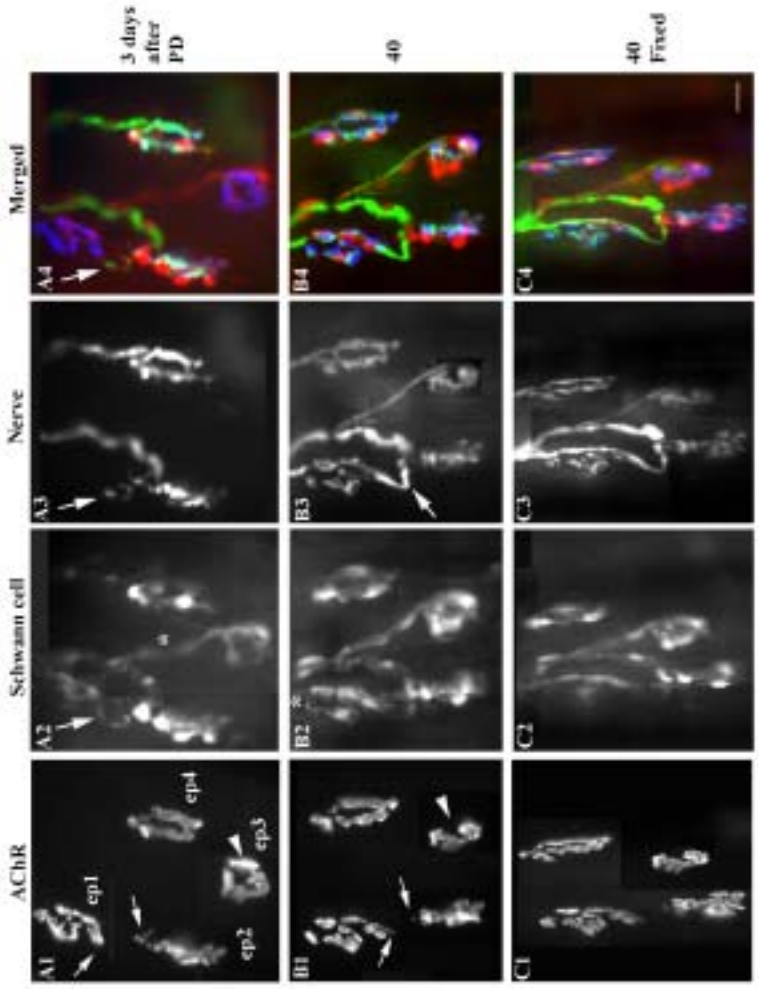

Figure 3.13: Loss of acetylcholine receptor sites occurs at endplates that extend or receive terminal sprouts but not at innervated endplates in which terminal sprouting is transitory.

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves at 4 endplates (ep1, ep2, ep3 and ep4) were viewed twice, 3 days and 40 days following partial denervation. In merged images (A4, B4, and C4), AChRs, SCs and nerves appear as blue, red and green, respectively. New rhodamine-bungarotoxin was applied on each day 3 and 40. When the images were collected 3 days following partial denervation, ep2 and ep4 remained innervated but ep1 and ep3 were denervated (A3). A terminal sprout had grown towards ep1 (arrow in A3 and A4) over the SC

bridge connecting ep1 and ep2 (arrow in A2). On day 40, this terminal sprout was found innervating ep2 (arrow in B3) and has grown in a retrograde direction in the old SC tube (asterisk in B2). Also on day 40, loss of AChR is found at the sites where the terminal sprout left ep2 (compare upper arrow in A1 and B1) and entered ep1 (compare arrows in ep1 in A1 and B1). Note also that loss of AChR sites (compare arrowhead in A1 and B1) is found at ep3 where a regenerating axon or a nodal sprout entered the endplate by growing along the old Schwann cell tube (asterisk in A2). In contrast, no loss of AChR sites is detected at ep4 (compare A1 and B1). The panels in C show the images obtained in vitro after sacrifice of the animal and removal and fixation of the muscles. The results in C agree with those obtained in vivo also obtained on day 40. Bar 20 μm

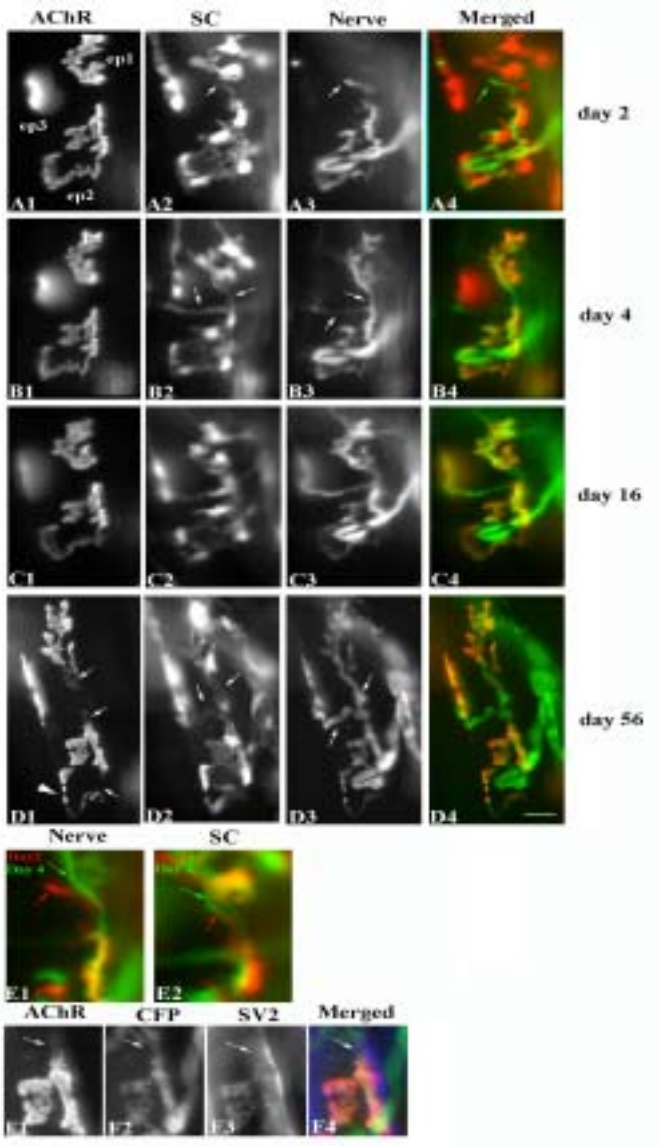

Figure 3.14: Acetylcholine receptors are lost from synaptic sites as a consequence of sprouting following partial denervation

Acetylcholine receptors (AChRs), Schwann cells (SCs) and nerves were viewed repeatedly at 3 endplates (ep1, ep2 and ep3) over the course of 56 days following partial denervation. In the merged images, SCs and nerves appear as red and green in A4, B4, C4 and D4, respectively. AChRs, nerves and synaptic vesicles

appear as red, blue and green in F4, respectively. In E1 and E2, both SCs and nerves on day 2 and day 4 appear as red and green, respectively. New rhodamine-bungarotoxin was applied on day 2, 16 and 56. On day 2 when the first images were collected, ep2 remains innervated, but the other 2 are denervated (A3). Already, a sprout has emerged from the top of ep2 (A3). However, the end of the terminal sprout (arrow in A3) is slightly beyond the SC process (arrow in A2). The terminal sprout is not oriented towards ep1 (green arrow in A4) but a part of the SC process appears to be (red arrowhead in A4). Images collected on day 4 show that both the SC process and the sprout have reached ep1 (right arrow in B2 and B3). To compare the changes in the SC processes and the sprout between day 2 and day 4, the panels E1 (showing the sprout) and E2 (showing the SC processes) were constructed. The images suggest that the top of the SC process keeps growing towards and contacts ep1 by day 4 (compare green and red arrows in E2). However, the sprout appears to withdraw back to the SC process and contacts ep1 by day4 (compare red and green arrows in E1). Additional growth is also seen in panel B on day 4: a sprout (left arrow in B3) has emerged about halfway between ep2 and ep1 and has grown towards ep3 in association with a SC processes (left arrow in B2). Sometime between day 4 and day 16, this sprout reinnervated ep3 (C3). These two sprouts and SC bridges from ep2 appear stable as they did not change over the course of 39-52 days. There is no detectable reinnervation of either ep1 or ep3 by any axon other than sprouts from ep2. Despite any obvious polyneuronal innervation, AChR sites are lost from the bottom and the top of ep2 and the bottom of ep1 where the terminal sprout leaves ep2 and enters ep1 (arrows in D1). These sites remain covered by axonal processes (arrow in F2). However, staining with anti-SV2 antibody suggests that these nerve processes lack significant accumulation of synaptic vesicle proteins (F3 and F4). There also appear to be changes in AChR on the bottom left of ep2 (arrowhead in D1). Bar 20 μm

that lacked persistent terminal sprouts and only one of these showed loss of AChR. An example is shown in Fig. 3.13.

Unlike AChR loss in muscles following nerve crush, receptor loss at innervated endplates appears to be relatively smaller on average 7.5 ±1.6% (n=9). This loss commonly occurred where terminal sprouts extend. However, receptor loss at the denervated endplates innervated by terminal sprouts (on average 17.5 ± 4.2 % (n=8)) is larger than the innervated endplates. The AChR sites at where the denervated endplates received terminal sprouts were lost in most cases. Therefore, we conclude that innervated and denervated endplates lose AChRs at sites where the terminal sprouts extend from and enter into endplates.

Other cases of AChRs loss and endplate remodeling following partial denervation

In addition to AChR loss taking place at denervated endplates reinnervated by terminal sprouts, I also found AChR loss occurred at endplates reinnervated by axons growing over old Schwann cell tubes. These axons could be nodal sprouts, terminal sprouts retrogradely growing or regenerating axons. A few cases further show this loss is associated with lack of occupation of AChR sites by Schwann cells following denervation and the failure of regenerating nerves to occupy these sites. An example is shown in Fig. 3.8.

Moreover, I also found persistent (n=3, 20 days after partial denervation) (Fig. 3.8) and temporary AChR addition (n=1, less than 20 days) at denervated endplates following partial denervation. All of these AChR additions took place along the pathway of Schwann cell processes and correlated escaped fibers.

DISCUSSION

We believe our results show the roles Schwann cells play during nerve sprouting in partially denervated muscles. Repeated imaging in transgenic animals allows us to observe activities of Schwann cells during nerve sprouting. In response to partial denervation, most of denervated endplates extend long Schwann cell processes; some of innervated endplates also extend relatively shorter Schwann cell processes. In some cases, the Schwann cell processes from denervated endplates form Schwann cell bridges interconnecting denervated and innervated endplates. These Schwann cell bridges are able to induce terminal sprouts to grow from innervated to denervated endplates, and then new synapses are formed. Once terminal sprouts interconnect two endplates, they are persistent in most cases. Moreover, these terminal sprouts frequently continue to grow over old Schwann cell tubes retrogradely and can even innervate endplates on the other ends of old Schwann cell tubes in some cases. Our experiments frequently show AChR loss in partially denervated muscles. Loss of AChR takes place not only at denervated endplates but at innervated endplates as most loss occurs long-term following partial denervation. Further observations show that this loss occurs most frequently at sites where endplates extend and receive terminal sprouts. Finally, my results also show that nodal sprouts arose from nodes and grew over old Schwann cell tubes.

Schwann cells extend processes in response to partial denervation.

In agreement with previous observations (Son and Thompson, 1995b; Love et al., 2003), I found that Schwann cells extended processes at denervated and innervated endplates following partial denervation. However, Schwann cell processes from denervated endplates were longer and more numerous. But what causes Schwann cells extend processes in partially denervated muscles? The stimulus for Schwann cell process formation could be produced by inactive muscles following denervation or by loss of contact between axons and Schwann cells (Brown et al., 1981; Sanes et al., 1986; Son and Thompson, 1995b). However, we believe that loss of the contact between axons and Schwann cells is the primary stimulus causing terminal Schwann cells to extend processes. First, recent studies showed that stimulation of partially denervated muscles did not alter the length or number of terminal Schwann cell processes despite a reduction in the number of Schwann cell bridges interconnecting denervated and innervated endplates (Love et al., 2003). This result can not be explained by production by inactive muscles causing Schwann cell process formation. Second, application of botulinum toxin produces extensions of Schwann cells on normal muscles; however these extensions are much smaller than processes at denervated endplates resulting from whole denervation or partial denervation (Son and Thompson, 1995b). Third, mature denervated Schwann cells are able to produce and release autocrine trophic factors which are necessary for their survival (Meier et al., 1999; Jessen and Mirsky, 1999). These trophic factors might also be involved in Schwann cell process formation. Overall, I suggest that denervation

stimulate Schwann cells to produce and release signal(s) that cause themselves and Schwann cells at innervated endplates to extend processes. The processes found at denervated endplates may be more robust due to gradients of signal(s) between denervated and innervated Schwann cells.

Schwann cells at denervated endplates extend processes towards innervated endplates and form Schwann cell bridges interconnecting them

In some cases, I found that Schwann cells extended processes towards adjacent endplates to form Schwann cell bridges. These Schwann cell bridges are formed by processes of the Schwann cells at denervated endplates in most cases. First, in double transgenic mice expressing GFP and CFP in Schwann cells and nerves respectively, I found that processes at Schwann cells at denervated endplates grew towards adjacent innervated endplates to form Schwann cell bridges. Further, results from Nestin-GFP mice expressing GFP only in denervated Schwann cells are consistent with these observations. These conclusions were consistent with previous observations from static images (Son and Thompson, 1995b). In a few cases, I also found that Schwann cell processes from innervated endplates are involved in formation of Schwann cell bridges resulting from both double transgenic and Nestin mice.

I did not find the specific orientation of Schwann cell process formation in all cases where there were denervated and innervated endplates occurred in a small area. However, we do not believe that Schwann cell processes grow randomly. There are several reasons for this. First, Schwann cell processes did not grow towards nearby innervated endplates in some our experiments. This can be

explained if we consider that the real distance between denervated and innervated endplates may be farther than what we expected from the microscopic images. Therefore, in these cases the interaction between innervated and denervated endplates might be too small to change the direction of the growth of Schwann cells processes from denervated endplates due to the long distance between them. Second, some of our in vivo results show that these processes grew crossing muscles fibers to form Schwann cell bridges despite that they initially grew along muscle fibers as they normally did in muscles following whole denervation. These observations suggest that Schwann cell processes were forced to change the direction of their growth during the formation of these Schwann cell bridges. Third, Love and Thompson (1999) found that most Schwann cell bridges were formed between innervated and denervated endplates resulting from static images in the muscles following partial denervation. Finally, previous observations showed blockade of synapse transmission by postsynaptic or presynaptic blockers inhibited Schwann cell bridge formation (Love and Thompson, 1999). Moreover, recent studies showed that stimulation of partially denervated muscles suppressed formation of Schwann cell bridges, but did not alter length or number of Schwann cell processes (Love et al., 2003). Therefore, they inferred that active muscles produced and released trophic signals to induce Schwann cell processes from denervated endplates to grow towards innervated endplates following partial denervation. Taken together, we suggest that innervated muscles produce and release signals that guide the growth of processes of denervated Schwann cells; however this guidance seems to work only over short distances. Frankly, it is hard

to evaluate the direction of the growth of Schwann cell processes after partial denervation. As described above, the real distance between two endplates is unknown. Moreover, Schwann cell bridges are formed quickly once Schwann cell processes grow towards innervated endplates. More detailed results may be obtained through time-lapsing imaging with much shorter intervals in the future.

Terminal sprouts grow along the pre-existing pathways: Schwann cell bridges to innervate adjacent endplates and continue to grow over old Schwann cell tubes retrogradely.

Our repeated vital images show that terminal sprouts grew along pre-existing Schwann cell bridges, in agreement with the interpretation of static images (Son and Thompson, 1995b). Following Schwann cell bridge formation, these bridges induce innervated endplates to extend terminal sprouts and then guide these sprouts to innervate denervated endplates. Therefore, Schwann cells have ability to induce and guide terminal sprouting in partially denervated muscles. In our observations, I never found terminal sprouts that were not accompanied by Schwann cell processes. Furthermore, in neonatal muscles, terminal Schwann cells quickly die by apoptosis following denervation (Trachtenberg and Thompson, 1996). Therefore, no terminal sprouts were found in neonatal muscles following partial denervation (Lubischer and Thompson, 1999). Taken together, these results suggest that Schwann cell processes are crucial for nerve terminal sprouting in partially denervated muscles.

However, only some of Schwann cell bridges are able to induce terminal sprouts in our experiments. In some cases, denervated endplates interconnected by Schwann cell bridges were reinnervated by other axons arriving over old

Schwann cell tubes. In a few of these cases, Schwann cell bridges induced small terminal sprouts but these sprouts were not persistent. These observations therefore suggest that Schwann cell bridges are not only substrates for terminal sprouts to grow, but also actively sense the changes at these endplates and thus change interactions between Schwann cells and axons. However, escaped fibers (regenerating axons extending beyond the endplate area) are able to innervate endplates that are already innervated by axons from old Schwann cell tubes in muscles following crush (in manuscript). This suggests initialization of terminal sprouting may be more difficult than the formation of escaped fiber.

Further, our observations also show that terminal sprouts are persistent, and frequently continue to grow over Schwann cell tubes rather than stop at denervated endplates in most cases. In a few cases, I was able to trace these terminal sprouts growing over old Schwann cell tubes and they innervated denervated endplates on the other ends of these tubes. I also found some of these terminal sprouts growing retrogradely over old Schwann cell tubes retracted long-term following partial denervation. These observations are consistent with our observations on nerve regeneration (in manuscripts). Why can terminal sprouts growing retrogradely be persistent or transient? We suggest that finding a target could be one reason for the formation of persistent retrograde growth. But this seems not able to explain all observations. First, I was not able to trace all terminal sprouts over old Schwann cell tubes; and thus some persistent terminal sprouts growing over old Schwann cell tubes might not reach other endplates. Second, in our previous experiments, soleus muscles were partially denervated by

resection of soleus nerves, leaving branches of tibial nerves intact in AO rats. In these rats, about 50% soleus muscles are innervated by two nerves: soleus nerves and branches of tibial nerves. To prevent nerve regeneration, two sides of soleus nerve stumps were ligated following resection. Stimulation of the soleus nerve stumps close to the soleus muscle was able to cause muscles to twitch (in manuscript; Thompson et al., 1977). Therefore, there may be additional reasons for the retrograde growth of nerve terminal sprouts.

Since terminal sprouts continue to grow over old Schwann cell tubes and Schwann cell bridges normally fail to induce terminal sprouting at reinnervated endplates, partial denervation probably produces little polyneuronal innervations. I found few cases of polyneuronal innervation despite some cases that I was not sure whether terminal sprouts or second axons were growing over the old Schwann cell tubes. These observations are consistent with previous observations (Luff et al., 1988).

Nodal sprouts are always accompanied by old Schwann cell tubes or Schwann cell processes.

Nodal sprouts are those sprouts that arise from nodes of Ranvier in injured muscles and more than one sprout can arise from the same node (Hoffman, 1950). Slack et al (1979) found that nodal sprouts normally arose from nodes close to where denervated sheaths branched and sprouts were absent from long length of intramuscular nerve that lacked such branch points although those lengths contained degenerated axons and passed close to denervated endplates (Brown et al., 1981). Our results are consistent with these observations. Because I am able to

repeatedly image these nodes and further expand our observations to Schwann cells, more information was obtained here. First, I found that nodal sprouts always grew over old Schwann tubes and there was no case where nodal sprouts were not associated with Schwann cells. Moreover, recent studies also showed that terminal Schwann cells quickly die by apoptosis following denervation but not Schwann cells in old Schwann cell tubes; no terminal sprouts but nodal sprouts were found in partially denervated neonatal muscles (Trachtenberg and Thompson, 1996; Lubischer and Thompson, 1999). Therefore, Schwann cells are important for nodal sprouting. Second, nodal sprouts were able to grow over old Schwann cell tubes retrogradely and towards denervated endplates as well; therefore no specific direction was there like escaped fiber growing over Schwann cell bridges in nerve regeneration. Third, in some cases, I found there were Schwann cell processes interconnecting nodes and old Schwann tubes. Further experiments showed these processes may grow from denervated Schwann cell tubes resulting from Nestin-transgenic mice. In some cases these nodes were very close to old Schwann tubes even aligned with them. These Schwann cell processes might not be detectable. Since the distances between old Schwann cell tubes and nodes extending sprouts are short, I was not able to know whether Schwann cell processes preceded nodal sprouts. However, resulting from old Schwann cell tubes guiding nodal sprouting as well as terminal Schwann cell processes inducing terminal sprouting, we suggest that Schwann cell processes from old Schwann cell tubes extend towards nodes and induce as well as guide the growth of nodal sprout.

Terminal sprouting causes AChR loss in partially denervated muscles

In muscles following nerve crush, there is AChR loss at sites where nerve terminals grow escaped fibers (in manuscript). Here, in partially denervated muscles I also found that AChR loss took place at sites where terminal sprouts extended from innervated endplates. So first what causes innervated endplates lose their receptors? Recent observations showed that transplanted nerves lacking axons (Schwann cell tubes) were able to induce host nerve terminal withdrawal, thus leaving AChRs uncovered (Trachtenberg and Thompson, 1997). This is consistent with our observations where Schwann cell processes extended towards innervated endplates and formed Schwann cell bridges. Therefore, we suggest that Schwann cell bridges formed by Schwann cell processes from denervated endplates induce withdrawal of terminals at innervated endplate. This will leave AChRs uncovered by nerve terminals and further cause loss of AChRs there. Moreover, I also found the loss of AChRs at denervated endplates reinnervated by sprouts. This can be explained by lacking of occupation by Schwann cells before nerve regeneration and withdrawal of nerve terminals following nerve regeneration discussed in Chapter 2.

It also raises another question: what is the potential mechanism that denervated Schwann cells "attack" innervated endplates and thus induce the withdrawal of nerve terminals? Neuregulin and its receptors may be involved in this process. Neuregulin-1 is the prototype of a family of factors that display homologies to EGF, and signal via receptor tyrosine kinases of the ErbB family.

Currently, four different neuregulin genes are known, the best characterised of which is neuregulin-1(Adlkofer and Lai, 1999). Two receptors, ErbB3 and ErbB4, bind Neuregulin-1 with high affinity. The functional neuregulin receptors appear, however, to be heteromers, in which ErbB2 is a component (Adlkofer and Lai, 1999; Alroy and Yarden, 1997). Heteromeric receptors are of primary importance in signaling of ErbB family members. The receptors of major importance in Neuregulin-1 signalling in vivo are ErbB3/ ErbB2, which signal during development of neural crest cells and Schwann cells, and ErbB4/ErbB2, which signal in the myocardium (Garratt et al., 2000). Direct axonal contact has been known for decades to induce proliferation of Schwann cells in ***vitro*** (Wood and Bunge, 1975; McCarthy and Partlow, 1976), and it has now been demonstrated that axon-associated (membrane-bound) NRG and Schwann cell associated ErbB2 are essential components of this signaling interaction [Morrissey et al., 1995]. ErbB3 and ErbB4 have also been shown to be expressed in Schwann cells in ***vitro,*** although the latter is only detectable by reverse transcription PCR (RT-PCR) (Syroid et al., 1995; Levi et al., 1995; Grinspan et al., 1996).

Recent observations showed that applications of GGF to muscles in development induced nerve terminal loss. These results were believed to be obtained through Schwann cells; and this nerve terminal loss was progressive and preceded changes in AChR density (Trachtenberg and Thompson, 1997). Moreover, ErbB2 and ErbB3, GGF receptors are expressed in Schwann cells during development (Garratt el at., 2000). In denervated muscles, the ErbB2, ErbB3 receptors were able to be detected in denervated Schwann cells

immunochemically; and furthermore expression of ErbB2/ErbB3 protein and mRNA, and GGF isoforms are up-regulated in response to sciatic axotomy beginning 5 d postaxotomy (Cohen et al., 1992; Carroll et al., 1997). Taken together, we suggest that interactions between denervated Schwann cells and nerve terminals might cause induction of the withdrawal of nerve terminal mediated by ErbB2 and ErbB3 receptors in muscles following partial denervation. Therefore, we infer the interaction between Schwann cells and axons is crucial for the stability of NMJs.

CHAPTER 4

Conclusion

Evidence from this dissertation provides further insight into the roles terminal Schwann cells and Schwann cells in the endoneurial tubes play in peripheral nerve regeneration. These experiments focused on induction and guidance of Schwann cells in nerve regeneration. First, terminal Schwann cells extend processes into perijunctional space in response to nerve injury and these processes will guide the formation of escaped fibers, when regenerated axons grow back to old synaptic sites over old Schwann cell tubes. In some cases, the Schwann cell processes form bridges interconnecting two endplates and these Schwann cell bridges will guide escaped fibers to reinnervate adjacent endplates, restoring their function. Polyneuronal reinnervation is formed when these endplates are reinnervated by second axons following old Schwann cell tubes. Interestingly, the escaped fibers reinnervating neighboring endplates, in some cases continue to grow retrogradely over the old Schwann cell tubes and even innervate endplates on other ends of these tubes. Therefore, this growth of escaped fibers guided by Schwann cell processes changes the nerve regeneration pattern. A summary cartoon is shown in Fig 4.1.

In my nerve regeneration experiments, most endplates rapidly extend long Schwann cell processes, and these processes are close to each. When axons grow back to endplates, Schwann cell bridges are formed very quickly due to the short distances between Schwann cell processes. Therefore, it is rarely possible to

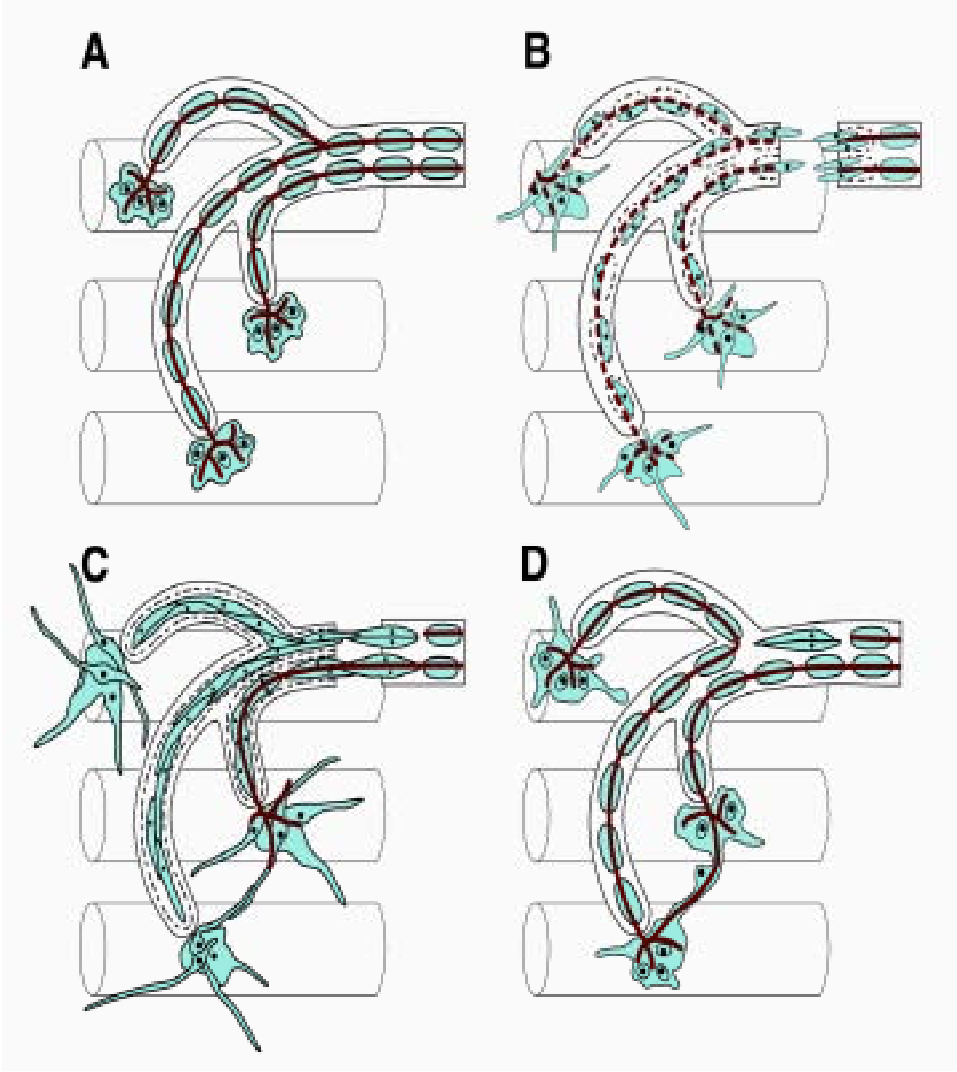

Figure 4.1: Cartoon depiction of the role of Schwann cells in the reinnervation of neuromuscular junctions.

Panel A depicts 3 muscle fibers and their innervation by myelinated axons in a branch of the intramuscular nerve. Axons are red; Schwann cells are blue. The top and the bottom muscle fibers are shown to be innervated by branches of the same motor axon. Panel B depicts the consequences of denervation; a resection of a piece of nerve is shown on the upper right. The myelin surrounded the axons distal to the site of injury is destroyed and the Schwann cells producing this myelin become activated and begin to extend processes up and down the

endoneurial tubes. Processes are extended from each end of the lesion site and will eventually grow to provide a pathway for axons to regenerate across the lesion. Similarly, terminal Schwann cells begin to extend processes away from the synaptic sites, but these do not yet "bridge" between synaptic sites. Panel C depicts an asynchronous reinnervation of synaptic sites that is commonly observed. An axon arrives at the lower site by following the endoneurial tube. As it reinnervates this site, Schwann cell bridges form with one of the neighboring sites. Panel D depicts the growth of an escaped fiber from the lower site, along the Schwann cell bridge, to reinnervate the adjacent site. The axon is shown continuing to grow in a retrograde direction up the endoneurial tube of the middle muscle fiber and then down the endoneurial tube of the upper muscle fiber to reinnervate this fiber as well. Schwann cells seem to function to provide a means of rapid reinnervation of all the synaptic sites in the muscle. Cartoon is modified from Son and Thompson (1995a).

observe how Schwann cell bridges are formed. However, partial denervation experiments allow me to do so. First, Schwann cell processes from denervated endplates are more robust than processes from innervated endplates, although both endplates extend processes following partial denervation. I am able to focus on Schwann cell processes from denervated endplates. Second, partial denervation is performed by resection on a portion of soleus nerves, so it takes long time for most of injured axons to regenerate. Therefore, in the muscles after partial denervation, a small area enclosing denervated and innervated endplates can be observed and denervated endplates remain denervated for a long enough time to allow me to observe how Schwann cell bridges are formed.

The second line of study in this dissertation deals with induction and guidance of nerve sprouting in muscles following partial denervation. Schwann cells at innervated and denervated endplates extend processes following partial denervation, but processes from denervated Schwann cells are more robust. Frequently, denervated endplates are reinnervated by axons from old Schwann cell tubes. These axons are from either sprouts growing over old Schwann cell tubes or regenerating axons from injury sites. In some cases, denervated endplates are reinnervated by terminal sprouts from adjacent innervated endplates. Additionally, these terminal sprouts frequently continue to grow retrogradely over old Schwann cell tubes of the denervated endplates as some escaped fibers do in nerve regeneration. Furthermore, these terminal sprouts were found to grow over Schwann cell bridges interconnecting denervated and innervated endplates. Further evidence shows that Schwann cells at denervated endplates extended long

processes towards adjacent innervated endplates and formed Schwann cell bridges, suggesting that there may be signals regulating Schwann cell process formation. Taken together, it is clear that Schwann cells have the ability to induce and guide nerve sprouting in partially denervated muscles. A summary cartoon is shown in Fig 4.2.

Schwann cell vs. AChR loss

Evidence from our nerve regeneration experiments shows that loss of AChRs is correlated with Schwann cell behaviour. As the nerve terminals degenerate following nerve injury, terminal Schwann cells extend processes from endplates and at the same time they gradually lose the coverage of a portion of AChRs. Since Schwann cells guide the growth of regenerating axons inside old synaptic sites, the portion of AChRs unoccupied by Schwann cells and their processes will be subsequently missed by regenerated axons. Finally, these AChRs are lost if the Schwann cells and regenerating axons do not re-occupy them later on. This abandonment of Schwann cells has also been noted in electron microscopic studies of denervated, mammalian junctions (Miledi and Slater, 1968). Results from Hyuno Kang's observations further show that the loss of Schwann cell occupancy is correlated with denervation time before nerve regeneration. Meanwhile, loss of Schwann cell occupation is also related to denervation time (in manuscript). Similar loss of receptor sites dependent on the timing of reinnervation was noted in the studies of Rich and Lichtman (1989; see

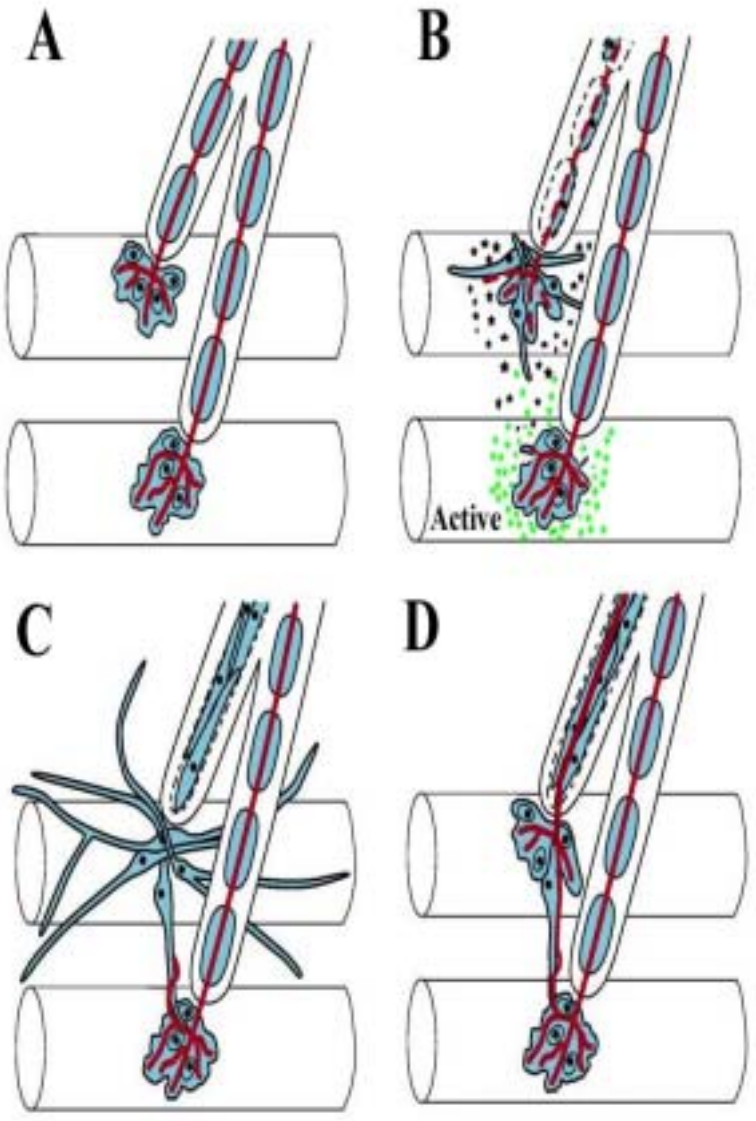

Figure 4.2: Cartoon illustrating the proposed role for Schwann cells in inducing and guiding nerve sprouting in partially denervated muscle.

(A) Two myelinated axons (in red) innervating two muscle fibers. Terminal Schwann cells (in blue) cover the nerve terminal processes at each junction. **(B)** Partial denervation of the muscle injures the axon that innervates the top muscle fiber. This axon begins to degenerate (indicated by dashed red line). The loss of

contact between terminal Schwann cells and nerve causes the NMJ to release some trophic factors (indicated by black stars). These factors induce the growth of long processes from the denervated endplate but shorter processes from the more distant SCs at the innervated endplate. Meanwhile, different trophic factors released by the NMJ remaining innervated attract the process which is closest to this innervated NMJ. In **(C)**, the process extended from the Schwann cells at the denervated junction on the top muscle fiber has reached the nerve terminal remaining on the bottom muscle fiber. The Schwann cell induces the nerve terminal on the bottom muscle fiber to sprout. In **(D)**, the nerve sprout has grown to reinnervate the denervated endplate and continued to grow over the old Schwann cell tube. Finally, the terminal Schwann cells here have begun to retract their processes. The consequence of these events, is that the denervated muscle fiber is now innervated by a sprout arising from the fiber that remained innervated following the partial denervation. Note that equally effective would be the withdrawal of a signal normally supplied to the SCs on the upper endplate by the nerve that suppresses SC growth. Cartoon is modified from Son and Thompson (1995b).

also (Holmes and Young, 1942). Taken together, loss of Schwann cell occupation partially accounts for AChRs lost following nerve regeneration.

These observations also raise two interesting questions. First, what is the direct cause of AChR loss, Schwann cells or nerves? We believe that lack of occupation of Schwann cells of AChRs gives axons a chance to determine which portion of the AChRs are lost. There are two reasons for it. 1. Even if a portion of AChRs is not covered by Schwann cells following denervation, no receptors loss occurs until the axons grow back. In the muscles following nerve resection, there is no receptor loss although the size of endplates becomes smaller due to atrophy of muscles (Rich and Lichtman, 1989; Balice-Gordon and Lichtman 1994). 2. Resection of nerve after muscle reinnervation can block this kind of receptor loss (Fig. 2.16). Taken together, after regenerating axons reach the old synaptic sites and follow Schwann cell processes to partially cover AChRs, interaction between inactive and active AChRs causes receptor loss unless Schwann cell processes and axons re-occupy these AChRs.

In some cases, Schwann cells and regenerating axons re-occupy AChRs that are abandoned by Schwann cells and nerves at the beginning of nerve regeneration. Love and Thompson (1998) found Schwann cells did proliferate after muscles were reinnervated. It is possible that the growth of Schwann cell processes is stimulated by regenerating axons growing back to old synaptic sites. Despite my observation of a few cases to support this idea, further experiments such as vital, repeated imaging with shorter intervals during nerve regeneration may clarify it in the future.

Additionally, I further found Schwann cells and regenerating axons withdrew after nerve regeneration and then caused AChR loss. In these cases, the AChRs were previously covered by Schwann cell process and regenerated axons. How does this happen? One possibility is that I missed some transient escaped fibers which caused polyneuronal reinnervation. Then, the loss of polyneuronal innervation led to AChR loss. However, I did not observe polyneuronal reinnervation in most cases where withdrawal of Schwann cell processes and nerve terminals caused AChRs loss. Moreover, recent observations showed several extracellular markers (NCAM, laminin α5, laminin β2, and VVA staining) remained for long times at sites of synapse elimination (Culican et. al., 1998). However, Schwann cell processes and regenerated axons are very dynamic, extending and withdrawing for long time following muscle reinnervation. Therefore, interaction between axons and Schwann cells might be one explanation. Schwann cells induce and guide nerve regeneration, and regenerating axons may regulate the activities of Schwann cells. Regenerating axons stimulate the growth of Schwann cell processes and at the same time Schwann cells may induce withdrawal of the nerve terminals inside endplates. Actually, AChR loss at innervated endplates in partially denervated muscles is also evidence that interaction between Schwann cells and axons can cause nerve terminals to withdraw and then lead to AChR loss.

Our observations clearly show that a single axon has the ability to cause AChR loss after nerve regeneration. So what happens in development? Recent evidence showed that, during NMJ development, axons took over the AChRs

which another axon previously innervated but lost in synapse elimination, and this taking over might not cause AChR loss (Walsh and Lichtman, 2003). Therefore, there might be another reason for AChR loss during development in addition to loss of polyneuronal innervation. It is possible that a single axon may be able to cause AChR loss and shape endplates in development. Meanwhile, Schwann cell might be involved this kind of AChR loss. However, the functions of Schwann cells in development are still unknown. Although previous observations showed that Schwann cells were unlikely to provide some guidance like in nerve regeneration, for example to ensure that motor neurons reach the correct muscles (Landmesser, 1980), there are conflicting results about the possibility that Schwann cells provide pathways that are then selected by the projecting neurons (Keynes et al., 1987; Noakes and Bennett, 1987; Dahm and Landmesser, 1988; Carpenter and Hollyday, 1992; Son and Thompson, 1995a). Finally, some recent results showed that Schwann cells were very dynamic when axons withdrew during synapse elimination (Lichtman, personal communication), suggesting Schwann cells may participate actively in synapse elimination.

Terminal Schwann cell vs. Schwann cell in endoneurial tubes

During development, Schwann cells differentiate from a common set of precursors into one of two morphologically and functionally distinct types: myelinating and non-myelinating Schwann cells (Jessen and Mirksy, 1991). This differentiation into `myelinating' and `non-myelinating' phenotypes is reversible. Upon loss of axonal contact after nerve resection, cells of both types de-

differentiate and express markers characteristic of an earlier developmental stage; these cells are termed reactive (Son et al., 1996). Denervated myelinating Schwann cells express many of the proteins that characterize terminal Schwann cells such as NCAM, L1, NGFR and GAP-43 (Curtis et. al., 1992; Daniloff et al., 1986; Jessen et al., 1987; Martini and Schachner, 1988; Taniuchi et al., 1986, 1988) and dramatically decrease their syntyhesis of myelinating related proteins and glycolipids (Gupta, 1993; Poduslo, 1993; Scherer and Salzer, 1996). Upon regeneration and restoration of axonal contact, these reactive cells reassume myelinating or non-myelinating phenotypes depending on the axons they contact (Son et. al., 1996). Our results concerning nerve regeneration show both terminal Schwann cells and Schwann cells in endoneurial tubes (myelinating Schwann cells) are able to guide regenerating axons. Furthermore, our observations on partially denervated muscles show or suggest that terminal Schwann cells and myelinating Schwann cells are able to induce and guide nerve terminal and nodal sprouting respectively. Taken together, terminal Schwann cells and myelinating Schwann cells may have common abilities to induce and guide nerve sprouting.

I also found most nodal sprouts occurred very close to endplates, in agreement with previous observations (Brown, 1981). This suggests that nodal sprouting or Schwann cell induction and guidance of this sprouting may require some signals from muscles or some other elements near muscles. Since our observations focused on the endplate area, I can not exclude the possibility that I might miss nodal sprouts forming far away from muscles. But at least it does occur very often in areas close to endplates. Despite similarity mentioned above,

terminal Schwann cells extend long Schwann cell processes in response to denervation but myelinating Schwann cells do not, despite small Schwann cell processes found between two close endoneurial tubes. This suggests that there may be barriers around endoneurial tubes to block myelinating Schwann cells from extending Schwann cell processes out of their tubes. We believe the biological meaning is to prevent the formation of the network of Schwann cell processes inside intramuscular nerves which could delay the axons to reinnervate endplates. Therefore, more axons are able to efficiently grow back to original endplates during nerve regeneration.

Are Schwann cells necessary for nerve sprouting?

Our observations of Schwann cell processes and nerve sprouting suggest that Schwann cells are crucial for nerve sprouting. Additionally, recent studies showed that denervation caused terminal Schwann cells apoptosis in muscles of neonatal rats and this apoptosis could be rescued by application of GGF (Trachtenberg and Thompson, 1996). There were no terminal sprouts following partial denervation in neonatal animals but nodal sprouts instead (Lubischer and Thompson, 1999). Such experiments suggest that the loss of terminal Schwann cells by apoptosis is correlated with an absence of nerve terminal sprouting. However, a better or direct way to investigate this would be to examine the possibility of nerve sprouting without Schwann cell presence. Attempts are underway in our lab to kill terminal Schwann cells using a laser micro beam.

Schwann cell process formation vs. reinnervation

Previous experiments also suggested that terminal Schwann cells could regulate the extent of the contact between the nerve terminal and the muscle fiber, perhaps by controlling the growth of the nerve (Trachtenberg and Thompson, 1997). It appears that a number of experiments indicate that actively growing axons, such as those during sprouting, are less efficient at forming efficacious synapses and lose efficacy even at synapses existing at the time of sprouting (Slack and Hopkins, 1982; Rochel and Robbins, 1988; Le and Thompson, unpublished results). This issue is an important one, because one of the consequences of degenerative diseases of motor neurons is the denervation of muscle fibers. Clearly Schwann cells appear to play an important role in the compensatory sprouting reactions that attempt to maintain/restore muscle strength. It may be the case that small amounts of sprouting are beneficial in this role, but that large quantities of sprouting actually weaken the ability of the nervous system to control muscle contractions (in manuscript).

Conclusion remarks

The goal of this work was to determine the roles Schwann cells play in response to nerve injury. Results presented in this dissertation provide direct evidence to show: Schwann cells extend processes in response to nerve injury and these processes guide escaped fibers and form Schwann cell bridges interconnecting two endplates during nerve regeneration; Schwann cell processes from denervated endplates grow towards innervated endplates and form Schwann

cell bridges which induce and guide terminal sprouts in partially denervated muscles. Furthermore, the portions of synaptic sites that lack Schwann cells are subject to a kind of elimination during reinnervation and its aftermath. Therefore, Schwann cells also are involved in AChR loss following nerve regeneration. Taken together, the results suggest that the Schwann cell is crucial for synapse formation and maintenance during nerve regeneration and therefore this work may have clinical relevance. Moreover, functions of Schwann cells shown here may suggest possible roles that other glial cells might play in CNS. Lastly, the observations of partial denervation experiments also suggest possible roles glial cells might play in synaptic plasticity.

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